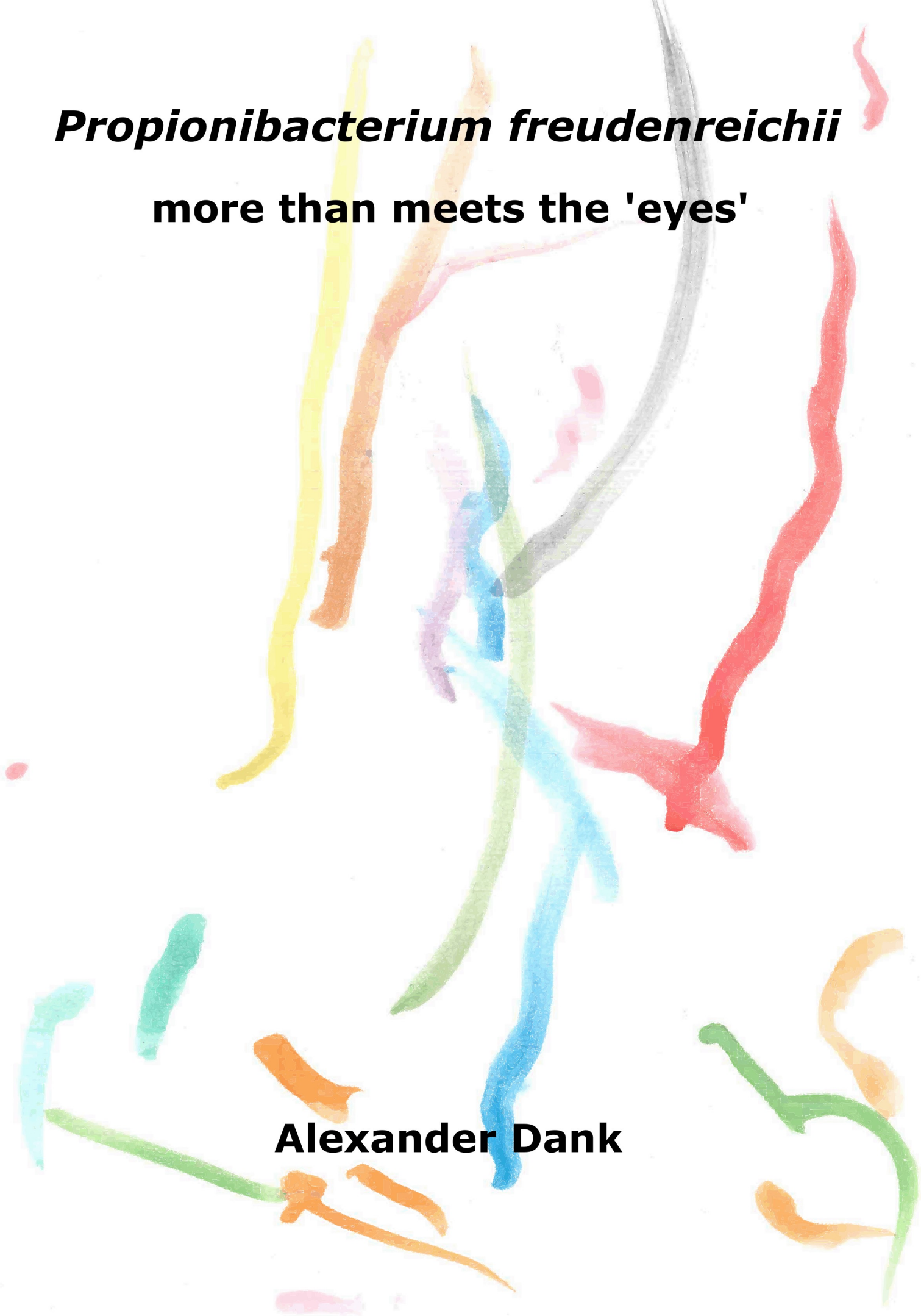
Propionibacterium freudenreichii

more than meets the 'eyes'



Alexander Dank

Propositions

1. *Propionibacterium freudenreichii* occupies a niche at the end of trophic chains.
   (this thesis)

2. Application of mixed bacterial and fungal starter cultures opens a window of opportunities for innovations of fermented foods.
   (this thesis)

3. The "Columbian Exchange" of food crops (Crosby, 1973) has benefitted Eurasia more than the Americas.

4. The fact that bees are able to discriminate between odd and even numbers (Howard *et al.*, 2022) indicates mathematical parity is evolutionary relevant.

5. The over-industrialization of our food system has led to significant decline of knowledge on artisanal food conservation and preparation methods in the general public.

6. It is the PhD candidate's responsibility to graduate on time.

Propositions belonging to the thesis, entitled:

Propionibacterium freudenreichii
more than meets the 'eyes'

Alexander Dank
Wageningen, 11^{th} of April, 2023

Propionibacterium freudenreichii more than meets the 'eyes'

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Propionibacterium freudenreichii more than meets the 'eyes'

Alexander Dank

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1 General introduction

A. Dank (2022)

Fermentation is one of the oldest techniques used by mankind to preserve food products. Many classical food products in the world like bread, cheese, wine, yoghurt, olives, vinegar, beer, tea, tempeh, soy sauce and many more are fermented (Allison and Macfarlane 1989). Fermentation is ubiquitously present in all cultures, which all use their own raw materials and microbiological workhorses to convert raw materials to the often so-typical products belonging to that culture. The periodic table of fermented foods by Gaenzle (2015) nicely shows this large variety of food fermentations carried out across the globe.

Although food fermentation has been performed for centuries, the actual role of the microbiological workhorses was not known until Louis Pasteur elucidated the roles of microbes in many fermentation processes (Bordenave 2003). From that point onwards, more and more research about the role of microorganisms in fermentation led to the isolation of single strains or mixed-cultures nowadays usually used as defined starter cultures. The use of defined starter cultures led to better product quality control and higher product consistency (Ross et al. 2002). In modern-day food industry fermentation processes are carried out on a large scale usually using these defined starter cultures.

Cheese production

One major group of fermented products produced throughout the world, with a production of 21.3 million tonnes in 2014, is cheese. The origin of cheese is believed to lie in the 'fertile Crescent', between the Tigris and Euphrates, in Iraq, some 8000 years ago together with the domestication of plants and animals (Fox et al. 2004). The importance of cheese is illustrated by the early notion of the product in several cultures; cheese is mentioned in the bible, in tombs in Egypt and in Greek and roman literature (Fox et al. 2004). The advantages of cheese being easier to transport and having a longer shelf life than milk must have helped with the spread of cheese production and development of a variety of techniques to produce it. Many cheese types being produced today are in fact originating from practices developed in monasteries, dating back to 1000 A.D. (Scott 1986).

In modern day cheese production it is common practice to add starter cultures to milk to start the acidification and hence cheese production. These starter cultures most often consist of lactic acid bacteria. For cheeses which are cooked to no higher temperatures than 40 °C a starter containing *Lactococcus lactis* and/or *L. cremoris* are normally used. For cheese varieties that are cooked to higher temperatures, e.g. Swiss-type and hard Itialian varieties, often mixed cultures of *Streptococcus thermophilus* and *Lactobacillus* spp. are being used (Fox et al. 2004). Growth of the starter cultures in milk results in consumption of lactose present in the milk which is primarily converted into organic acids, like lactate and acetate, which consequently lowers the pH of the milk to a point at which coagulation of casein proteins occurs, resulting in the formation of a gel. Next to coagulation, metabolic activities of microbial cheese communities also results in significant aroma and organic acid production (Engels et al. 1997), providing the typical desired organoleptic properties. An elaborate overview of cheese production and all different types of cheese is given by Fox et al. (2004). In Swiss-type cheese some of the lactate that is produced by lactic acid bacteria is consumed by propionic acid bacteria (PAB), which convert this lactic acid into propionic acid, acetic acid and CO_2. The production of CO_2 consequently causes the formation of the characteristic 'eyes' in this cheese variety (Fox et al. 2004). Growth of propionibacteria is essential for the taste and aroma development of several cheese varieties, like Swiss-type (Allison and Macfarlane 1989; Thierry et al. 2004) and Leerdammer (Britz and Riedel 1994).

Propionibacteria

Propionibacteria are Gram-positive, non-spore forming bacteria belonging to the group of actinobacteria with a high GC-content (53-68%) (Pophaly et al. 2012). Propionibacteria belong to the family of *Propionibacteriaceae* and were historically classified based on their habitat as cutaneous or dairy propionibacteria. However, later Scholz and Kilian (2016) proposed to reclassify propionibacteria based on whole-genome sequence analysis of 162 species of the family *Propionibacteriaceae* by addition of three novel genera, *Acidipropionibacterium, Cutibacterium* and

Pseudopropionibacterium respectively. Major species of dairy propionibacteria that are isolated mainly from milk, cheese, dairy products and rumen are *P. freudenreichii, A. acidipropionici, A. jensenii* and *A. thoenii* (Pophaly et al. 2012).

P. freudenreichii was the first dairy species isolated from Emmental cheese, and is responsible for the 'eyes' present in the cheese by its fermentation of lactate into propionate, acetate and CO_2 via a metabolic pathway called the Wood-Werkman cycle (Falentin et al. 2010). Propionibacteria are mainly studied for three different purposes; the production of cheese (Allison and Macfarlane 1989; Engels et al. 1997; Fox et al. 2004; Scott 1986), the production of propionic acid (Kaspar 1982; Navone et al. 2018) and the production of cobalamin (vitamin B_{12}) (Burgess et al. 2009; Quesada-Chanto et al. 1998; Wolkers–Rooijackers et al. 2018). Recently, also the prebiotic/probiotic potential of propionibacteria is studied. *P. freudenreichii* showed good survival capabilities in the upper intestinal tract (Huang and Adams 2004), is able to produce bifidogenic factors (Mori et al. 1997) and displays immunomodulatory capacity (Rabah et al. 2018), making this microbe a promising prebiotic and probiotic.

To exploit the biotechnological potential of propionibacteria it is relevant to understand their ecological niche. This thesis focusses on four different aspects relevant for the biotechnoligcal application of *P. freudenreichii*; I) Increasing biomass production, relevant for the production of starter/adjunct cultures used during vitamin B_{12} or cheese production by studying aerobic respiration in *P. freudenreichii*. II) The utilisation of substrates found in the gut, aiming at improving knowledge about the potential surivival of *P. freudenreichii* in the human gastro-intestinal tract and thus their potential as probiotic. Here the focus lies on 1,2-propanediol and ethylene glycol, which are (1,2-propanediol) or may be (ethylene glycol) produced by other microbiota in the gastro-intestinal tract. III) The production of vitamin B_{12} in a biotechnological setting. Here the focus lies on the potential of low amounts of oxygen to stimulate vitamin B_{12} production in *P. freudenreichii* . IV) Application of *P. freudenreichii* in novel food products for *in situ* B_{12} enrichment.

Fermentation and respiration

In order to maintain cellular processes and in order to grow, bacterial cells oxidise substrates to release electrons. These electrons can be used to perform work or can be stored in high energy containing compounds, like ATP, that can be utilised later. In this process electrons are carried by electron carriers, like NAD^+, which are reduced (NADH). In order to continue oxidation of the substrate these redox-carriers must be regenerated. Fermentation and respiration are two different solutions to solve this redox problem.

Fermentation is defined as the oxidation of an organic compound while using endogenous electron acceptors, usually catabolic intermediates of the same organic compounds. Respiration is defined as the oxidation of an organic compound while using extracellular electron acceptors, via a functioning electron transport chain (ETC) that can generate a proton motive force (PMF) (Brooijmans 2008). Consequently, respiration releases more energy as substrates can both be degraded to further extend and during degradation a proton motive force is created that allows the cell to perform work or store this energy in ATP through oxidative phosphorylation. Next to oxygen, a wide variety of external electron acceptors can be used by bacteria. In general, the components needed for a functional electron transport chain consist of dehydrogenases, a quinone pool and a terminal oxidase. A good overview of electron transport chains, (anaerobic) respiration, the components and mechanisms of these systems is described by Brooijmans (2008).

Fermentation in propionic acid bacteria

Fermentation of most substrates in propionic acid bacteria results in the production of propionic acid, hence the name of the bacteria. As substrates are oxidised to acetate and CO_2, NAD^+ is reduced to NADH. This NAD^+ needs to be regenerated by donating electrons to an electron acceptor. In anaerobic conditions propionic acid bacteria regenerate NAD^+ by the Wood-Werkman cycle, resulting in the production of propionate. Propionic acid bacteria use a partial (reversed) tricarboxylic acid

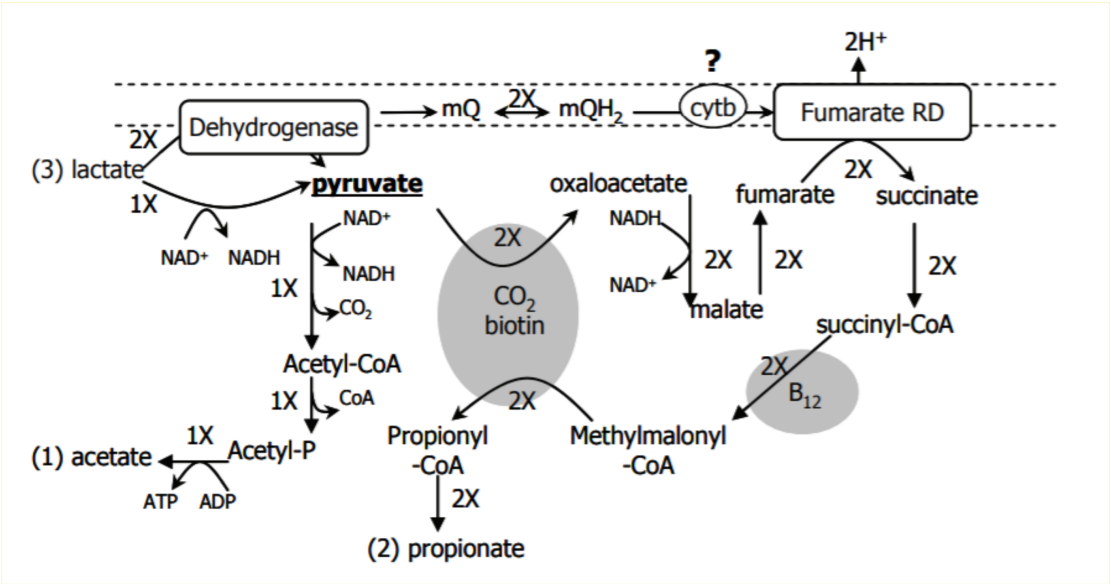

Figure 1.1: A simplified overview of the Wood-Werkman cycle (from Brooijmans 2008)

(TCA) cycle for this in combination with a few key enzymes present in the Wood-Werkman cycle, methylmalonyl-CoA carboxyltransferase, methylmalonyl-CoA epimerase and methylmalonyl-CoA mutase (Falentin et al. 2010). A simplified version of this cycle is displayed in Figure 1.1.

The oxidation of 1 mol of lactate to 1 mol of acetate reduces 2 moles of NAD^+. The production of propionate oxidises 1 mol of NADH to NAD^+. Hence, in order to be redox balanced, for each mol of lactate oxidised to acetate, two moles of propionate need to be produced. The reaction stoichiometry therefor is: 3 lactate --> 2 propionate + 1 acetate + 1 CO_2 (Brooijmans 2008). The oxidation of lactate to acetate produces one mol of ATP through substrate-level phosphorylation, whereas the conversion of lactate to propionate generates proton motive force using fumarate as an electron acceptor (Figure 1.1). This pathway yields per 3 mol of lactate 1 mol of ATP though substrate-level phosphorylation (acetate production) and translocates 2 H^+ per propionate produced. Depending on the assumed stoichiometry of ATPase of 3 H^+ per ATP or 4 H^+ per ATP (Bott and Niebisch 2003), 2x 0.5-0.67 ATP is generated through oxidative phosphorylation (Seeliger et al. 2002). The total theoretical ATP yield per mol of lactate is thus 0.67-0.78 mol ATP/mol lactate.

Respiration systems in propionic acid bacteria

The Wood-Werkman cycle relies largely of enzymatic functions also present in the tricarboxylic acid cycle (TCA). And even though the genus is generally recognised as anaerobic to aerotolerant, the complete TCA cycle and genes required for respiratory pathways and electron transport chains were found in *Acidipropionibacterium acidipropionici* (Parizzi et al. 2012) and *P. freudenreichii* (Falentin et al. 2010). Aerobic respiration has also been experimentally validated in propionic acid bacteria (Cove et al. 1987; Gent-Ruijters et al. 1976). Next to aerobic respiration, also anaerobic respiration has been shown for nitrate (Allison and Macfarlane 1989; Kaspar 1982) and humic acid (Benz et al. 1998) and complete combustion of lactate, glycerol and propionate to CO_2 was observed for *P. freudenreichii* in a poised-potential amperometric culture system (Cove et al. 1987). It is therefore not surprising that *P. freudenreichii* contains all components for a functional electron transport chain. NADH dehydrogenase, menaquinone, cytochrome bd-complex, ATPase and the complete pathway for heme synthesis were found to be encoded in the genome of *P. freudenreichii* (Falentin et al. 2010).

P. freudenreichii DSM20271 was shown to be able to oxidise acetate through a modified citric acid cycle (Beck and Schink 1995). The modified citric acid cycle suggested by Beck and Schink (1995) generates 3 NAD(P)H and 1 menaquinol per cycle from acetyl-CoA. Isocitrate dehydrogenase (1.1.1.42) generates 1 NADPH, succinate semialdehyde dehydrogenase (2.6.1.9) generates 1 NADPH, succinate dehydrogenase (1.3.5.1) generates menaquinol, and malate dehydrogenase generates 1 NADH (1.1.1.37).

Several propionic acid bacteria contain d-,b-,o- a and c- type cytochromes (Brüggemann et al. 2004; Schwartz and Sporkenbach 1975). The number of protons transported across the membrane per electron transferred over an electron transport chain (H^+/e^-) is three for the cytochrome bc_1-aa_3 complex ($3H^+/e^-$) and one for cytochrome bd oxidase ($1H^+/e^-$) (Bott and Niebisch 2003). Some propionic acid bacteria also have been shown to be able to generate proton motive force through

NADH dehydrogenase, a menaquinone pool, cytochrome c reductase and aa3-type cytochrome (Brooijmans 2008). If assuming protein motive force can be generated using a type-1 NADH dehydrogenase and cytochrome c (aa_3) oxidase, the transfer of 2 electrons from NADH to oxygen would translocate 8 H^+ (Bott and Niebisch 2003). When assuming the terminal oxidase to be cytochrome bd-type the transfer of 2 electrons would translocate $4H^+$. Assuming ATPase requires 3 to 4 protons to be translocated per synthesis of ATP, the theoretical yield of this respiratory chain would be 2-2.67 ATP per NADH generated for an bc_1-aa_3 type chain and 1 to 1.33 ATP for a bd-type chain (Bott and Niebisch 2003). The presence or absence of specific terminal oxidase types therefor greatly affects the potential yield of biomass cells can gain per consumption of substrate.

Although the potential energetic gain of using oxygen as terminal acceptors, propionic acid bacteria are commonly referred to as anaerobic or aerotolerant. Indeed, their relationship with oxygen is complex and reports about the effect of oxygen on growth and survival are contradicting. Based on genomic information the status as anaerobe would not be expected. Indeed, Schwartz and Sporkenbach (1975) already concluded based on their analysis of electron transport in *P. freudenreichii* that propionic acid bacteria cannot be considered to be anaerobes, as their electron transport chains were very much a like those found in (micro)aerobes. However, propionic acid bacteria are not able to grow on plates aerobically and showed lower growth rates in prolonged aerobic conditions (De Vries et al. 1972; Ye et al. 1996), due to decreased cytochrome synthesis and thus loss of electron transport chain integrity (De Vries et al. 1972). The complex role of aerobic electron transport chains in propionic acid bacteria therefor yet needs to be elucidated.

Propionibacteria as probiotics

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al. 2014). Probiotic bacteria are selected on the criteria that they are safe, can survive the digestive tract and may confer a health effect (Altieri 2016). Recently, probiotic potential was reviewed

for several dairy propionic acid bacteria (Cousin et al. 2011). Dairy propionibacteria have a long history of safe use in Swiss-type cheese and therefore have the "generally recognised as safe" (GRAS) status (Pophaly et al. 2012), opening up the potential as probiotic. The digestive tract survival was assayed by Campaniello et al. (2015), who showed propionibacteria are able to survive acid stress (pH 2.5 for 3 hours) and bile salts. *In vivo* survival of *P. freudenreichii* in the human digestive tract was validated by Suomalainen et al. (2008) and *P. freudenreichii* has been isolated from a breast-fed human infant (Colliou et al. 2017). Survival and colonization of the intestines requires the ability to metabolize carbon sources present in such ecosystems. One such carbon source is 1,2-propanediol, which is a major end product from anaerobic degradation of rhamnose or fucose by human intestinal microbiota. 1,2-Propanediol can be metabolized by propanediol-degrading bacteria into propionate, ATP and 1-propanol maintaining redox balance. Anaerobic metabolism of 1,2-propanediol requires the production of toxic intermediate propionaldehyde. Some bacteria protect themselves from this toxic intermediate by encapsulating the enzymatic processes in self-assembling proteinaceous organelles called bacterial microcompartments (Axen et al. 2014; Sampson and Bobik 2008). The cluster encoding these bacterial microcompartments (**P**ropane**D**iol **U**tilisation cluster (*pdu*)) has been identified in *P. freudenreichii* and was shown to be expressed in the intestines of a pig (Saraoui et al. 2013). However, Saraoui et al. (2013) did not show active metabolism of 1,2-propanediol by *P. freudenreichii* nor whether expression of the *pdu* cluster results in assembly of bacterial microcompartments in *P. freudenreichii*. The presence and potential role of bacterial microcompartments in *P. freudenreichii* thus remains to be elucidated.

Role of vitamins as co-factors in metabolic processes

Vitamins are micronutrients that are essential for the functioning of metabolic processes. Many vitamins cannot be synthesised by humans themselves and thus must be acquired through diet. Propionic acid bacteria can synthesise most vitamins

de novo (Van Wyk et al. 2018), with the exception of pantothenate and biotin for all strains and thiamine and *p*-aminobenzoic acid for some strains (Falentin et al. 2010). *P. freudenreichii* DSM20271 requires only pantothenate, biotin and thiamine for growth (Borghei et al. 2021).

One of the key vitamins produced by propionic acid bacteria in high quantities is vitamin B_{12}. Vitamin B_{12} plays an important role in DNA synthesis in humans and deficiency can lead to various malfunctioning, like megaloblastic anemia, growth retardation and neurological problems (Dhonukshe-Rutten 2004). In bacteria, vitamin B_{12} is essential for enzymatic functions involved in carbon rearrangement reactions (for instance isomerisation of succinyl-CoA to methylmalonyl-CoA), dehydration reactions (for instance 1,2-propanediol dehydratase), deamination of aminoalcohols and DNA synthesis (reduction of ribonucleotide triphosphate to 2-deoxyribonucleotide triphosphate) (Vorobjeva 1999).

In propionic acid bacteria vitamin B_{12} is involved in large variety of processes. The characteristic Wood-Werkman cycle requires cobalamin (vitamin B_{12}) as a cofactor of methylmalonyl-CoA mutase (EC 5.4.99.2) for the isomerisation of succinyl-CoA to methylmalonyl-CoA. There is also indirect evidence for involvement in methionine synthesis, stabilisation of thiol groups in enzymes and methylation of cytosine residues and glutamate mutase activity (Vorobjeva 1999). Vitamin B_{12} is coupled to production of DNA and RNA and therefore required for biomass formation (Iordan and Petukhova 1995). Furthermore, the 1,2-propanediol dehydratase encoded in the *pdu* cluster is B_{12} dependent (Bobik et al. 1999).

Many other vitamins are important in the metabolism of propionic acid bacteria. Biotin is a cofactor in methylmalonyl-CoA carboxytransferase (EC 2.1.3.1), which converts methylmalonyl-CoA and pyruvate into propanoyl-CoA and oxaloacetate in the Wood-Werkman cycle. The fermentation of lactate and respiration of substrates requires a functioning electron transport chain with a menaquinone pool (vitamin K_2) (Brooijmans 2008). The synthesis of methionine and DNA requires folate (vitamin B_9) (Hugenholtz et al. 2002; Vorobjeva 1999) and folate also plays a role in the glycine cleavage pathway, that may be used for additional energy generation

in propionic acid bacteria (Navone et al. 2018). Furthermore riboflavin (B_2) is involved in electron transport chains as FAD and FMN (Vorobjeva 1999; Zárate 2012). As shown, these vitamins all play key roles in fermentation and/or respiration processes. Switching the mode of metabolism, the requirements of the cells for each vitamin changes and thus also the production may change, as was shown for menaquinone in *Lactococcus lactis* (Liu et al. 2019) and vitamin B_{12} in propionic acid bacteria (Ye et al. 1996). Elucidation of the effect of fermentation or respiration as a mode of energy generation and the effect on vitamin B_{12} production therefor shows potential for vitamin production yield improvements and needs to be further explored. Furthermore, differences in growth substrates altering catabolic enzymatic requirements, such as induction of B_{12}-dependent diol dehydratases, may also be a suitable strategy to produce vitamin B_{12}.

Thesis outline

The main focus of research on propionic acid bacteria is performed on anaerobic conditions in relation to biotechnological production of cheese, propionic acid or vitamins.

In this thesis I describe studies of *P. freudenreichii* in conditions shedding more light on its biological niche and I attempt to use these conditions to implicate possible improvements in biotechnological applications. This thesis will cover 4 main topics: I) the complex role of propionic acid fermentation and respiration with focus on its physiological role, II) the effect of different modes of metabolism on growth and vitamin B_{12} production, III) The potential role of bacterial microcompartments in *P. freudenreichii* and its involvement in metabolism of 1,2-propanediol and ethylene glycol, IV) the application of *P. freudenreichii* and other microorganisms in novel fermented food product development. The thesis will conclude with a general discussion.

In **chapter 2** a screening of 16 dairy isolate propionibacteria for biomass formation was performed in anaerobic and aerobic conditions, yielding significantly higher biomass for most strains in aerobic conditions. *P. freudenreichii* DSM20271

was tested for optimum oxygen concentration for biomass production and grown in a prolonged aerated batch culture. This revealed that in specific aerated conditions propionibacteria first metabolise lactate into propionate and acetate, then consume propionate and eventually consume acetate in a respiratory fashion. We coined the switch from propionate production to consumption 'the propionate switch'. Proteome analyses showed reversibility of the Wood-Werkman cycle in this process in combination with activation of proteins in electron transport chains. Our results thereby reject the general description of propionic acid bacteria as anaerobes.

In **chapter 3** the role of bacterial microcompartments in degradation of 1,2-propanediol, an important substrate in the gut, is investigated. Our results show that *P. freudenreichii* contains bacterial microcompartments that enable the degradation of 1,2-propanediol by protecting the cell against toxic intermediates produced during metabolism of this substrate. These bacterial microcompartments therefor may play an essential role in survival and colonisation of the gut by *P. freudenreichii* and could be crucial for the probiotic potential of *P. freudenreichii*.

In **chapter 4** the role of bacterial microcompartments in degradation of ethylene glycol (1,2-ethanediol), a potential metabolic end product of xylan degradation in anaerobic environments, is investigated. Our results indicate that the *pdu* cluster enables metabolism of ethylene glycol in *P. freudenreichii* by expression of bacterial microcompartments, leading to formation of ethanol and acetate and additional biomass formation. Based on our study and other studies we discuss the potential dual function of the *pdu* cluster in metabolism of 1,2-propanediol and ethylene glycol.

In **chapter 5** vitamin B_{12} production using the aerobic electron transport chain on lactate and propionate was assayed and compared to anaerobic vitamin B_{12} yield on lactate. Vitamin B_{12} yield was found to increase on lactate and this could be largely contributed to the phase in which propionate was being oxidised. Using propionate as sole carbon source increased the vitamin B_{12} yield even further. Both yield per substrate as yield per gram cell dry weight significantly increased using microaerobic oxidation of propionate.

In **chapter 6** nutritional enhancement and novel fermented food product development by cross-over fermentations are discussed. Cross-over fermentations are fermentation processes in which a microorganism is taken from one food product and is applied to a novel substrate and/or partner to produce nutritionally enhanced fermented food or novel fermented food products.This is examplified by a case study of *P. freudenreichii* for *in situ* fortification of B_{12} in lupin milk fermentation and a case study of *Aspergillus oryzae* enhancing aroma in a fermented dairy product. An outlook of cross-over fermentation concepts is given for novel fermented food products.

In **chapter 7** the results of this thesis are discussed together with relevant literature to provide a synthesis of all current findings and an outlook for future research.

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2

Propionibacterium freudenreichii thrives in microaerobic conditions by complete oxidation of lactate to CO_2

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Summary

In this study we show increased biomass formation for 4 species of food-grade propionic acid bacteria (*Acidipropionibacterium acidipropionici, Acidipropionibacterium jensenii, Acidipropionibacterium thoenii* and *Propionibacterium freudenreichii*) when exposed to oxygen, implicating functional respiratory systems. Using an optimal microaerobic condition, *P. freudenreichii* DSM 20271 consumed lactate to produce propionate and acetate initially. When lactate was depleted propionate was oxidised to acetate. We propose to name the switch from propionate production to consumption in microaerobic conditions the 'propionate switch'. When propionate was depleted the 'acetate switch' occurred, resulting in complete consumption of acetate. Both growth rate on lactate (0.100 h^{-1} versus 0.078 h^{-1}) and biomass yield (20.5 versus 8.6 g/mol lactate) increased compared to anaerobic conditions. Proteome analysis revealed that the abundance of proteins involved in the aerobic and anaerobic electron transport chains and major metabolic pathways did not significantly differ between anaerobic and microaerobic conditions. This implicates that *P. freudenreichii* is prepared for utilizing O_2 when it comes available in anaerobic conditions. The ecological niche of propionic acid bacteria can conceivably be extended to environments with oxygen gradients from oxic to anoxic, so-called microoxic environments, as found in the rumen, gut and soils, where they can thrive by utilizing low concentrations of oxygen.

Introduction

Propionic acid bacteria (PAB) are Gram-positive, non-spore forming bacteria belonging to the group of actinobacteria with a high GC-content (53-68%) (Poonam et al. 2012). PAB belong to the family of *Propionibacteriaceae* and were historically classified based on their habitat as classical (cutaneous) or dairy PAB. However, later Scholz and Kilian (2016) proposed to reclassify classical and dairy PAB based on whole-genome sequence analysis of 162 strains of the family *Propionibacteriaceae* by addition of three novel genera: *Acidipropionibacterium, Cutibacterium* and *Pseudopropionibacterium*. Major species of dairy PAB that are isolated mainly from milk, cheese, dairy products and rumen are *Propionibacterium freudenreichii, Acidipropionibacterium acidipropionici, Acidipropionibacterium jensenii and Acidipropionibacterium thoenii* (Poonam et al. 2012).

Notably, PAB can be found in a large variety of environments, ranging from the deepest cave of the world (Kieraite-Aleksandrova et al. 2015), soil (Hayashi and Furusaka 1979), silage (Merry and Davies 1999), human skin (Perry and Lambert 2006) and other tissue (Perry and Lambert 2011), cheese (Britz and Riedel 1994) and the rumen of animals (Bryant 1959). This variety of environments points to versatility in metabolic traits of which the repertoire can be extended by the capacity to use a range of terminal electron acceptors. Indeed, PAB have been reported to use several alternative electron acceptors, like nitrate (Allison and Macfarlane 1989; Kaspar 1982) and humic acid (Benz et al. 1998) next to oxygen (Gent-Ruijters et al. 1976). The complete TCA cycle and genes corresponding to respiration pathways and electron transport chains were found in *Acidipropionibacterium acidipropionici* (Parizzi et al. 2012) and *Propionibacterium freudenreichii* (Falentin et al. 2010). Although their uses are mainly in anaerobic processes, the presence of all genes required for respiration and fully functional electron transport chains (Falentin et al. 2010; Merry and Davies 1999) shows the potential for applications in (micro)aerobic biotechnological processes. However, studies addressing the effect of oxygen on propionic acid bacteria present contradicting results. An increased growth rate

and biomass production is reported for *P. freudenreichii* (Cardoso et al. 2004; Quesada-Chanto et al. 1998), showing the potential benefit of using oxygen as a terminal electron acceptor. However also the inability of *P. freudenreichii* to grow aerobically on agar plates, lower growth rates (Vries et al. 1972; Ye et al. 1996) and decrease of cytochrome synthesis and consequent loss of electron transport chain integrity are reported under aerobic conditions (Vries et al. 1972), which shows the sensitivity of *P. freudenreichii* to high levels of oxygen. Oxygen toxicity can be combatted by PAB by expressing superoxide dismutase, catalase and cytochrome c oxidase, although distribution of these enzymes seem to be species specific (Cove et al. 1987). The relationship of various species of propionic acid bacteria with oxygen is thus complex and requires further study.

The aims of this study are twofold. First we determined biomass formation and metabolite production of food-grade PAB strains as a function of various exposure to oxygen. The food-grade PAB strains are represented by the species *A. acidipropionicii, A. jensenii, A. thoenii, P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii*. Secondly, the common used dairy isolate *P. freudenreichii* DSM 20271 was selected for an in-depth study of its response to oxygen. An optimum oxygen flux was determined in chemostat cultivations and using this oxygen concentration the biomass production, primary metabolite production and proteome were monitored during a long term cultivation. Our study provides implications for PAB ecology as well as industrial applications including increased biomass production and yield enhancements for efficient production of PAB adjunct cultures and/or for various biotechnological purposes, like vitamin and aroma production.

Results and discussion

Screening of response to oxygen for sixteen propionic acid bacteria

In this study we screened the response to various levels of oxygen for sixteen PAB strains (supplementary file 1). Biomass formation (optical density at OD_{600nm})

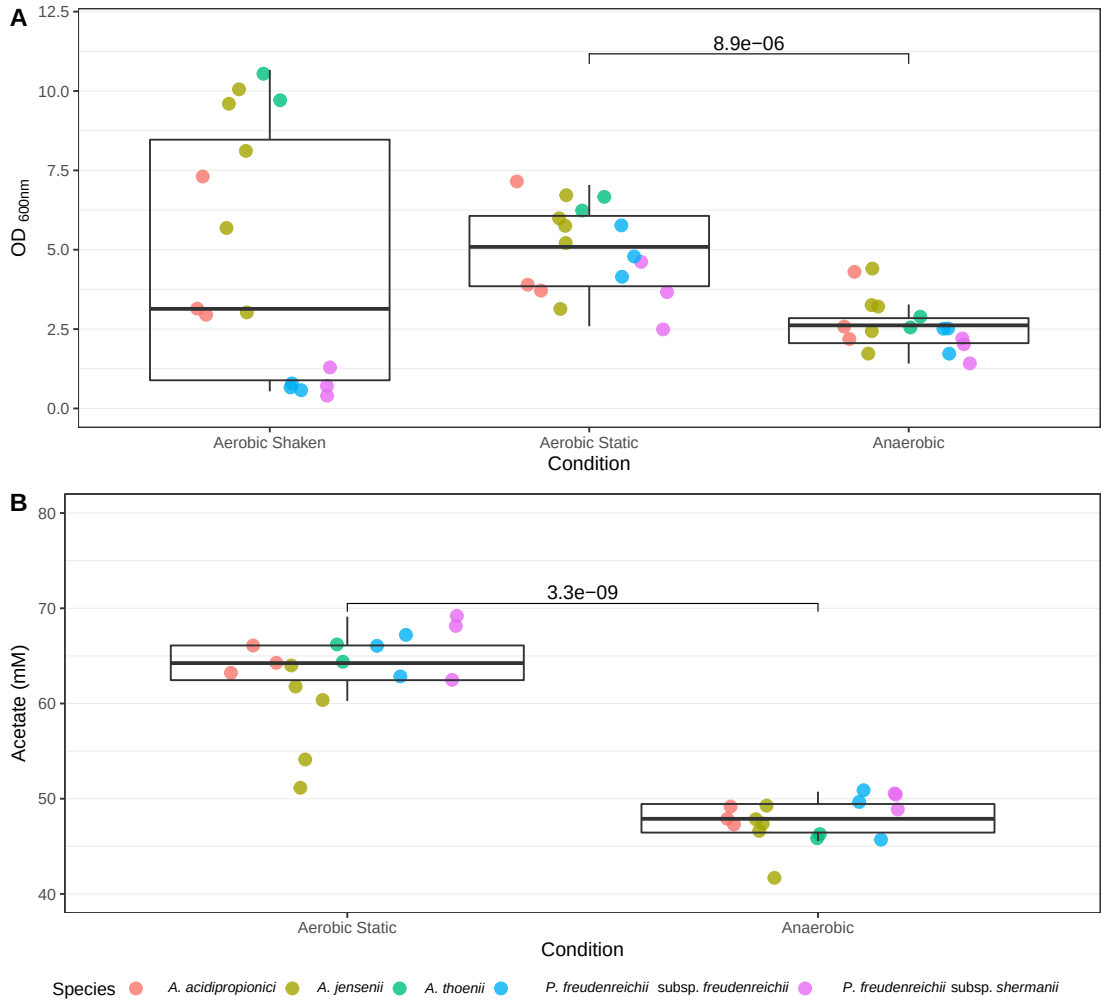

Figure 2.1: Biomass production **(A)** and acetate production **(B)** for four species of propionic acid bacteria of cells growing in aerobic shaking (120 RPM), aerobic static and anaerobic conditions. Each data point represents biological duplicates for individual strain (n=16 strains, biological duplicates.)

was determined for each strain after 5 days of incubation in yeast extract lactate medium in three conditions: (i) aerobic shaking (120 RPM), (ii) aerobic static and (iii) anaerobic. Anaerobic cultivation resulted in a mean OD-value of 2.6 ± 0.2, aerobic static in a mean OD of 5.0 ± 0.3 and aerobic shaken in a mean OD of 4.8 ± 1.0 (Fig. 2.1A). A significant difference between the OD-values was found between anaerobic and aerobic static ($p < 1E^{-5}$, Pairwise Wilcoxon test).

The availability of oxygen enhanced biomass formation in static conditions for all strains, although the strain-to-strain variation was enlarged. In aerobic shaken conditions the increased oxygen levels resulted in lower biomass production of

the selected *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* strains compared to anaerobic conditions. Several *A. acidipropionici*, *A. thoenii* and *A. jensenii* strains were able to produce similar or higher biomass compared to anaerobic conditions and thus are more tolerant to higher oxygen levels.

Metabolite formation was determined for aerobic static and anaerobic conditions. The presence of oxygen resulted in a significantly higher amount of acetate being produced by strains incubated in aerobic static conditions ($p < 1e^{-8}$, Wilcoxon rank sum test) compared to anaerobic conditions (Figure 2.1B).

Higher acetate production in aerobic conditions provides evidence that active electron transport using oxygen as terminal electron acceptor is widespread in propionic acid bacteria. The higher biomass formation shows that propionic acid bacteria can energetically benefit from respiratory electron transport, in line with the genomic information available for *P. freudenreichii* (Falentin et al. 2010) and *A. acidipropionicii* (Parizzi et al. 2012). However, our results also clearly demonstrate the toxicity of high oxygen levels for certain PAB in aerobic shaking conditions.

Inter and intra-species differences in the aerotolerance of PAB may be explained by variability in oxygen-defence systems (Cove et al. 1987), hence each individual strain must have an optimal level of oxygen at which it can combat oxygen toxicity and have maximal energetic benefit. We focussed further on studying the effect of oxygen on the commonly used cheese adjunct culture *P. freudenreichii* DSM 20271, which obtained an OD_{600} of 4.0 in aerobic static conditions and an OD_{600} of 0.9 in aerobic shaken conditions, clearly showing both the growth stimulation effect of O_2 and its toxicity at higher exposure. In order to find the optimal oxygen concentration for *P. freudenreichii* DSM 20271, we performed chemostat cultivations at pH 7.0 at a constant dilution rate of 0.1 h^{-1}, while varying the amount of oxygen supplied to the system. The highest concentrations of biomass were formed using oxygen supplies of 4.2 and 8.4 ml $O_2 * L^{-1} * min^{-1}$. At oxygen supplies above 8.4 ml $O_2 * L^{-1} * min^{-1}$ the biomass formation showed a sharp decline (Supplementary figure 1). In further experiments an oxygen supply of 6.3 ml $O_2 * L^{-1} * min^{-1}$ was used to study the growth, metabolite production and proteome of *P. freudenreichii* DSM 20271.

Microaerobic conditions increase biomass production in prolonged batch cultivations by complete oxidation of lactate

Metabolite production and biomass formation

P. freudenreichii DSM 20271 was cultured in a prolonged batch cultivation in pH and temperature controlled bioreactors for a period of 21 days. Biomass production, primary metabolite production (figure 2.2) and proteomes were monitored.

In anaerobic conditions lactate was consumed within 72 hours and a stable propionate:acetate ratio of 2.1:1 was found throughout the duration of the cultivation after lactate depletion. Without additional external electron acceptors *P. freudenreichii* is thus unable to further degrade these organic acids.

In microaerobic conditions lactate was completely oxidised in three distinguishable phases, i) Lactate was consumed and propionate and acetate were produced ii) propionate was oxidised to acetate iii) acetate was completely oxidised to CO_2 (figure 2.2). Oxygen was actively consumed throughout the cultivation, as dissolved oxygen levels dropped below the detection limit 1 day after inoculation and remained undetectable until all propionate was consumed. Oxygen levels also remained undetectable during acetate consumption, but increased again towards the end of the acetate consumption phase, signifying a reduction in respiration rate (supplementary figure 2). Metabolite production and biomass formation in each phase is discussed below.

Lactate consumption phase In microaerobic conditions, lactate was consumed within 46 hours and propionate and acetate were formed in a ratio of 1.56:1. A significantly higher maximum growth rate (0.100 ± 0.002 h^{-1} versus 0.078 ± 0.004 h^{-1}, one-tailed t-test, $P<0.01$) and higher biomass (OD_{600} of 3.7 ± 0.5 versus 2.9 ± 0.6, one-tailed t-test, $P<0.05$) was achieved in the lactate consumption phase (phase 1) for cells grown microaerobically compared to anaerobically-grown cells. Anaerobic fermentation of lactate resulted in an average yield of 8.6 ± 1.2 g cell dry weight/mol lactate (table 2.1), in line with the yield of 8.1 g cell dry weight/mol

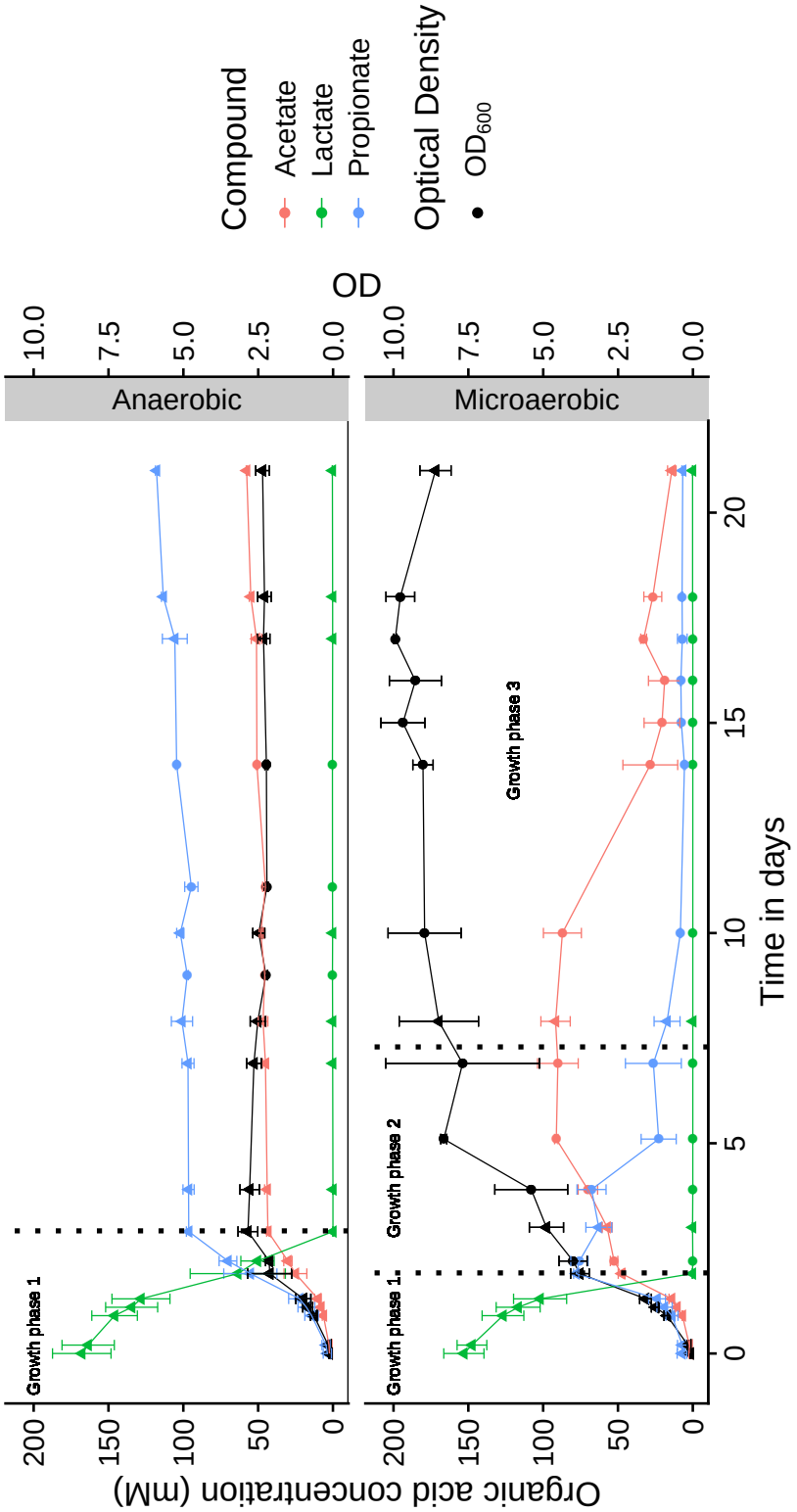

Figure 2.2: Metabolite production and biomass production of *P. freudenreichii* DSM 20271 during cultivation under anaerobic and microaerobic conditions over a course of 21 days. Three clear phases can be distinguished: Growth phase 1 : Lactate consumption and propionate and acetate production. Growth phase 2: Propionate conversion to acetate and CO_2. Phase 3: Acetate oxidation to CO_2. Error bars present the standard error of biological replicates. Biological replicates are displayed by either triangles (n=3) or circle (n=2).

Table 2.1: Calculated cell dry weight, summation of cell yield after each phase and maximum growth rate of *P. freudenreichii* grown in anaerobic or microaerobic conditions.

Consumption phase	Cell dry weight (g/kg)	Yield (g CDW/mol lactate)	Maximum growth rate (h^{-1})
Microaerobic Lactate consumption phase	1.8 ± 0.4	11.6 ± 0.7	0.100 ± 0.001
Microaerobic Propionate consumption phase	3.1 ± 0.10	20.5 ± 2.3	0.008 ± 0.003
Microaerobic Acetate consumption phase	3.1 ± 0.03	20.3 ± 1.8	N.D.
Anaerobic lactate consumption phase	1.4 ± 0.02	8.6 ± 1.2	0.078 ± 0.004

lactate reported by Vries et al. (1973) for anaerobic growth on a synthetic medium but low compared to the yield of 10.2 on complex lactate medium.

Differences in yeast extract content may account for the different yield reported by Vries et al. (1973). Microaerobic conditions yielded 11.6 ± 0.7 g cells/mol lactate, a 1.3 fold increase compared to anaerobic conditions. The production of propionate in microaerobic conditions implies that the flux through the electron transport chain is limited or stoichiometrically constrained by the availability of oxygen, resulting in propionate production as main electron sink.

Propionate consumption phase As cells deplete their lactate pool, the secreted organic acids become a potential source of energy in the presence of external terminal electron acceptors. When lactate was depleted, a switch from production to consumption of propionate was observed in microaerobic conditions (phase 2). Propionate was depleted after 143 hours and an increase of biomass from OD_{600} 3.7 ± 0.5 to 9.2 ± 1.1 was observed. Growth rates in the propionate consumption phase decreased to 0.008 ± 0.003 h^{-1}. Microaerobic conditions yielded an average estimated biomass of 20.5 ± 2.3 g cell dry weight*mol^{-1} lactate after exhaustion of propionate, a 2.4 fold increase compared to the yield observed on lactate in anaerobic conditions.

A switch from propionate production to consumption was also observed by Ye et al. (1996) after changing from anaerobic to aerobic conditions in *P. freudenreichii*. *Escherichia coli* switches from production towards dissimilation of secreted acetate ('the acetate switch') when its preferred substrate becomes limiting and electron transport is possible (Wolfe 2005). In analogy to this 'acetate switch', we propose

to name the change from production to consumption of propionate when lactate is depleted in presence of electron acceptors in *P. freudenreichii* the 'propionate switch'.

Acetate consumption phase Interestingly, addition of diluted hydrochloric acid to control the pH in the bioreactor continued from this point onwards, indicating consumption of organic acids. Indeed acetate consumption was observed after depletion of propionate (phase 3). Acetate was consumed and no other organic acids were detected, indicating complete oxidation to CO_2. Beck and Schink (1995) showed *P. freudenreichii* DSM 20271 is able to completely oxidise acetate through a modified citric acid cycle using hexacyanoferrate as electron acceptor, supporting our findings. The oxidation of acetate was not accompanied by a further increase in $OD_{600nm,}$ in line with results of Beck and Schink (1995) who found linear growth kinetics and a maximum biomass increase of two. The linear growth kinetics observed on acetate could explain the preferred order of the 'propionate switch' with production of acetate instead of complete combustion of propionate immediately. A schematic overview of the consumption phases based on proteome analysis and literature of *P. freudenreichii* metabolism (discussed below) is shown in figure 2.3.

In order to metabolise lactate, propionate and acetate as carbon sources, these compounds have to be transported into the cell. The undissociated form of these acids are membrane-permeable (Salmond et al. 1984; Saparov et al. 2006) by passive diffusion. In our cultivations (pH 7.0) the main form of the acids is dissociated and these require active transport systems. The presence of a transporter of propionate and/or acetate would greatly increase the ability to grow on these substrates in environments with neutral pH. L-lactate and D-lactate can be transported into the cell by lactate permease (lldP) (Núñez et al. 2002), which has also been identified in the genome of *P. freudenreichii*. For propionate and acetate we did not find any annotated transporters in the genome of *P. freudenreichii*. Monocarboxylate uptake systems for propionate and acetate have been described for several other bacteria (Ebbighausen et al. 1991; Fernández-Briera and Garrido-Pertierra 1988; Gimenez et al. 2003; Hosie et al. 2002; Reed et al. 2006) and yeast (Casal et al.

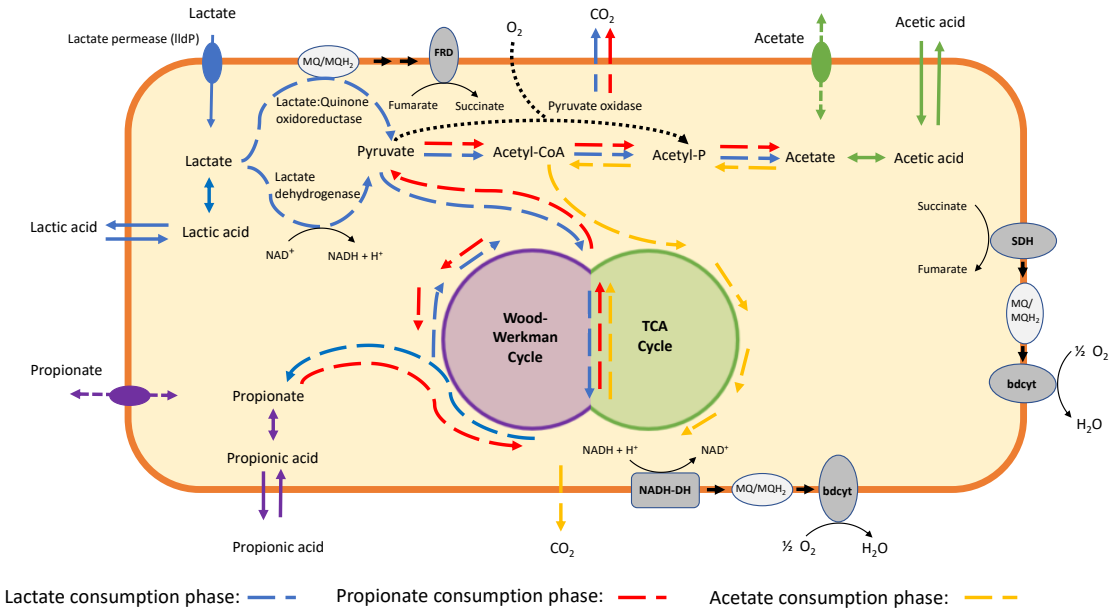

Figure 2.3: Schematic overview of the metabolism of lactate in *P. freudenreichii* in microaerobic conditions. Undissociated acids can be transported through passive diffusion (open arrows across membrane). Lactate is metabolised in three phases: a lactate consumption pase, propionate consumption phase and acetate consumption phase. In the lactate consumption phase lactate is metabolised to propionate and acetate. In the propionate consumption phase 'propionate switch' occurs and the produced propionate is metabolised to acetate. In the acetate consumption phase the 'acetate switch' occurs and acetate is metabolised to CO_2. Several options have been proposed for the production of Acetyl-CoA from pyruvate. Either a pyruvate dehydrogenase complex (producing NADH) or pyruvate ferredoxin oxidoreductase (producing reduced ferredoxin) catalyse the conversion of pyruvate to Acetyl-CoA. Recently, a ferredoxin based energy conservation system has been suggested in propionibacteria (Marcellin et al. 2020) consisting of FixABCX, which oxidises reduced ferredoxin and a quinol by reducing 2 NAD^+ (Ledbetter et al. 2017; Marcellin et al. 2020). The entire complex (FixABCX) could also be reconstituted in our proteomics data, supporting the possible role of pyruvate-ferredoxin oxidoreductase in the synthesis of Acetyl-CoA from pyruvate and maintaining redox balance for anaerobic growth on lactate. A pyruvate oxidase (black dotted line), producing acetyl-phosphate from pyruvate and phosphate while consuming oxygen has also been identified in the genome of *P. freudenreichii*, which may function as an energy yielding oxygen tolerance mechanism (Marcellin et al. 2020) and which may explain lower propionate:acetate ratios found in microaerobic conditions compared to anaerobic conditions in *P. freudenreichii*. Two gene clusters of the succinate dehydrogenase/fumarate reductase can be found in the genome of *P. freudenreichii*, *sdhABC1* (RM25-1246, RM25-1247, RM25-1248) and *sdhA3B3C2* (RM25-1350, RM25-1351, RM25-1352). Most likely, one copy of the succinate dehydrogenase genes acts as fumarate reductase whilst the other copy acts as succinate dehydrogenase (Brzuszkiewicz et al. 2011; Parizzi et al. 2012). FRD = fumarate reductase, SDH = succinate dehydrogenase, bdcyt = bd-type cytochrome, NADH-DH = NADH dehydrogenase, MQ = menaquinone, MQH_2 = menaquinol

1996). Blasting JEN1, a high-affinity symporter of lactate, pyruvate and acetate in *Saccharomyces cerevisiae*, resulted in a protein hit with 46% homology in *P. freudenreichii* annotated as Ydjk, a sugar (and other) transporter of unknown function which was not detected in our proteome analysis. In order to elucidate whether or not active transporters for propionate and/or acetate are present in *P. freudenreichii*, studies on the uptake rates of these monocarboxylates at different pH values need to be performed. This also further elucidates the effect of pH on the growth performance of *P. freudenreichii* on these monocarboxylates in the presence of external electron acceptors.

Bioenergetics

A functional respiratory chain greatly affects ATP production per substrate, as shown by the increased biomass yield in microaerobic conditions. Anaerobic growth on lactate using the Wood-Werkman cycle yields per 3 lactate, 1 ATP by substrate-level phosphorylation and 2x 2/3 ATP by fumarate reduction (Seeliger et al. 2002). This totals to a maximum ATP yield of 0.78 mol ATP per mol lactate.

The modified citric acid cycle suggested by Beck and Schink (1995) generates 3 NAD(P)H and 1 menaquinol per cycle from acetyl-CoA. Assuming lactate is converted to pyruvate by L-lactate:menaquinone oxidoreductase (1.1.5.12), generating menaquinol, and pyruvate entering the TCA through acetyl-CoA using pyruvate dehydrogenase (1.2.4.1/2.3.1.12/1.8.1.4), generating 1 NADH, a total yield of 4 NAD(P)H and 2 menaquinol per lactate is calculated. The number of protons translocated across the membrane per electron transferred over an electron transport chain (H^+/e^-) consisting of a type-I NADH dehydrogenase, a menaquinone pool and cytochrome bd oxidase is two ($2H^+/e^-$) per NADH oxidised, one by type-I NADH dehydrogenase (Bongaerts et al. 1995) and one by the bd-type cytochrome (Bott and Niebisch 2003). Oxidation of menaquinol via bd-type cytochrome results in one proton translocated per electron (1 H^+/e^-). Since 2 electrons are transferred in NADH oxidation and 2 electrons are transferred in menaquinol oxidation a total of 20 protons can be translocated per lactate. Succinate:menaquinone reductase

requires a proton gradient to reduce menaquinone, which lowers the total potential ATP production by protons with 2 translocated protons that cannot be used by ATPase (Schirawski and Unden 1998). A total of 18 protons per lactate oxidised are calculated. Assuming ATPase requiring 3 protons per ATP (Bott and Niebisch 2003) a theoretical ATP yield of 6 ATP per mol lactate oxidised is calculated. Complete oxidation of lactate would thus maximally increase the ATP yield by a factor of 7.7 compared to the ATP yield by fermentation.

Our experimental results showed an increase in biomass of 2.4-fold of cells in microaerobic conditions compared to anaerobic conditions, well within the limits of the maximum theoretical energetic advantage and similar to the biomass increase of 2.7 found by Pritchard et al. (1977) in chemostat conditions. The theoretical increase in biomass based on ATP generation difference is much higher (7.7 times). Partly, this may be explained by expression of pyruvate oxidase (Marcellin et al. 2020). Our proteomics data showed pyruvate oxidase is expressed in anaerobic and microaerobic conditions and low oxygen contents did not significantly change expression. If pyruvate oxidase is used as an oxygen consuming defence mechanism in microaerobic conditions instead of pyruvate dehydrogenase or pyruvate ferredoxin oxidoreductase in anaerobic conditions, less reducing equivalents (one less NADH) are produced and the theoretical ATP yield is lowered by 1.33 ATP. However, the theoretical yield when assuming pyruvate oxidase activity is still much higher. Beck and Schink (1995) report sub exponential growth kinetics and as a consequence low biomass yields when acetate is oxidised by *P. freudenreichii*. We hypothesise that because of these sub exponential growth kinetics, generated energy from acetate oxidation is mainly directed towards maintenance processes.

Proteome analysis of long-term batch cultivation

A proteomic analysis of cells in anaerobic and for each consumption phase in microaerobic conditions was performed. In total 1326 unique proteins were identified amongst all samples after strict filtering. For all identified proteins Volcano plots were prepared comparing the consumption phases and identifying significantly

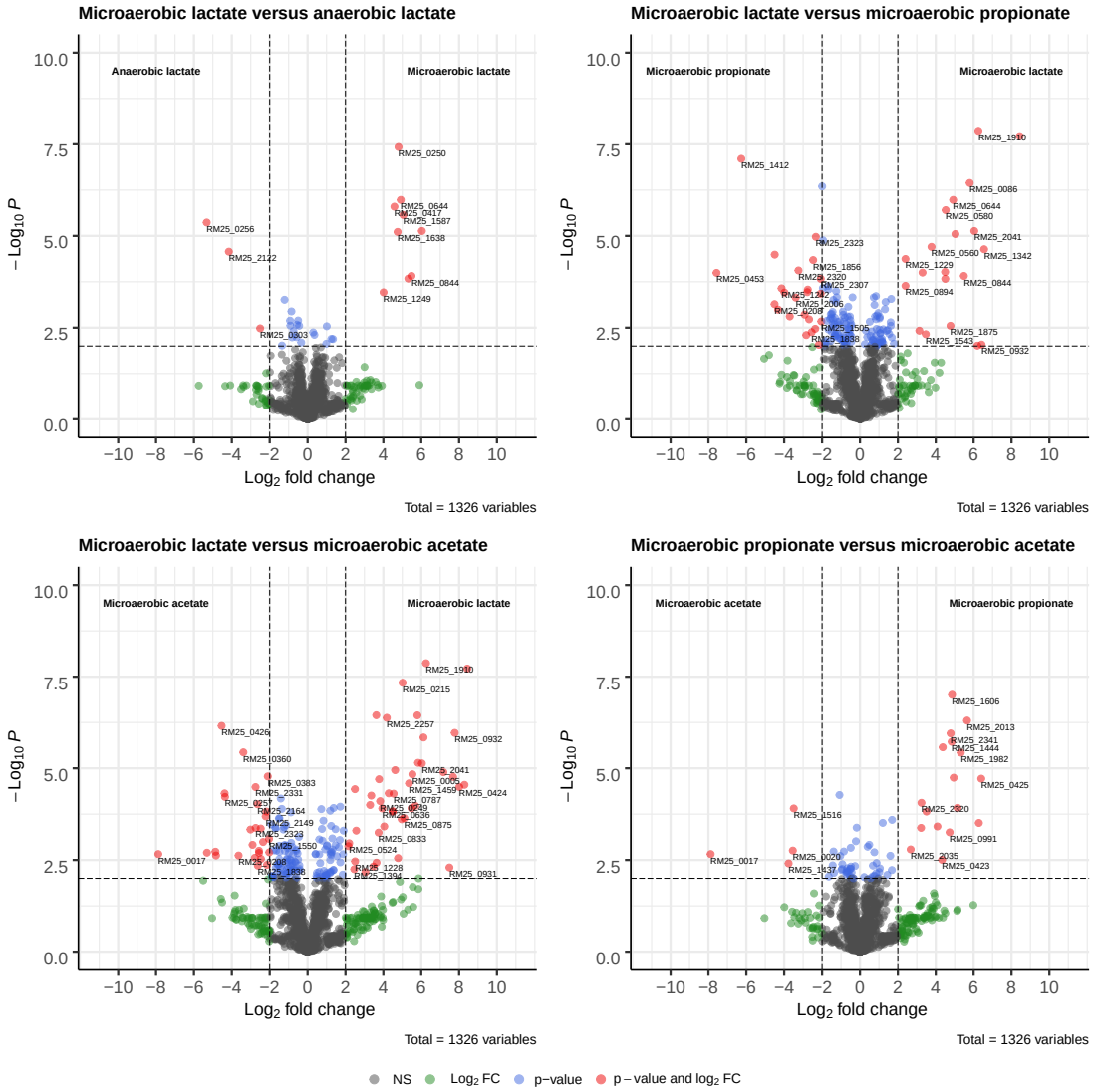

Figure 2.4: Volcano plots of proteomes measured during anaerobic and microaerobic growth conditions. A comparison is made between two phases: Reference versus treatment, positive log 2 fold-change values indicate higher abundance in reference sample, negative log 2 fold-change values indicate higher abundance in compared sample. Each point represents a unique protein. Colours represent significant differences between reference and treatment. Grey: Not significant (NS); green: >4-fold difference; blue: P<0.01; red: >4-fold difference and P<0.01 A list of significant proteins for each comparison is available in supplementary file 2.

different abundant proteins (4-fold increase and p<0.01 (figure 2.4)). A list with all significantly differentially expressed proteins for all conditions can be found in supplementary file 2.

To determine proteome similarity amongst samples and reveal protein expression patterns across samples we clustered the samples using hierarchical clustering and

K-means partitioning (MacQueen 1967). Hierarchical clustering was used to cluster samples based on proteome similarity. K-means partitioning was applied with k=8 for identification of clusters of proteins with similar expression patterns across the samples (Karimpour-Fard et al. 2015) (figure 2.5, supplementary file 3). Cluster 2,3,4 and 6 did not show specific patterns amongst hierarchical grouped samples and thus most likely consists of proteins that are continuously expressed and which expression patterns were based on random variance across individual samples. These clusters were not used for further analysis of protein expression. Cluster 1,5 and 8 did show expression patterns correlated to the hierarchical grouping, indicating these protein expression patterns are clustered based on the differential expression in the different consumption phases and thus biologically relevant.

Anaerobic and respiratory pathways are continuously expressed in anaerobic and microaerobic conditions

In both anaerobic and microaerobic conditions expression of the complete pathways for metabolism of lactate to propionate and acetate were detected. We detected L-lactate permease (*lldP*), lactate dehydrogenase (NADH dependent) (*ldh1, ldh2*) and a complex of 3 proteins which has been linked to lactate oxidation coupled to fumarate reduction by quinones or to cytochromes (Lactate utilization protein (*lldE, lldF, lldG*)) (Pinchuk et al. 2009).

All proteins in the Wood-Werkman cycle were detected. Pyruvate-flavodoxin oxidoreductase (*nifj1*), pyruvate oxidase (RM25_0410) and pyruvate dehydrogenase (*aceE, pdhB, bkdA1*) were found, together with phosphate acetyltransferase (*pta*) and acetate kinase (*ackA*) completing the pathway for acetate production. Interestingly, proteins involved in the Wood-Werkman cycle were as abundant in the propionate consumption phase compared to that in lactate consumption phase, and no upregulation of other enzymes known to be involved in bacterial propionate metabolism were found, supporting the hypothesis of the Wood-Werkman cycle running in reversed direction as the pathway for propionate oxidation, as previously suggested by Emde and Schink (1990).

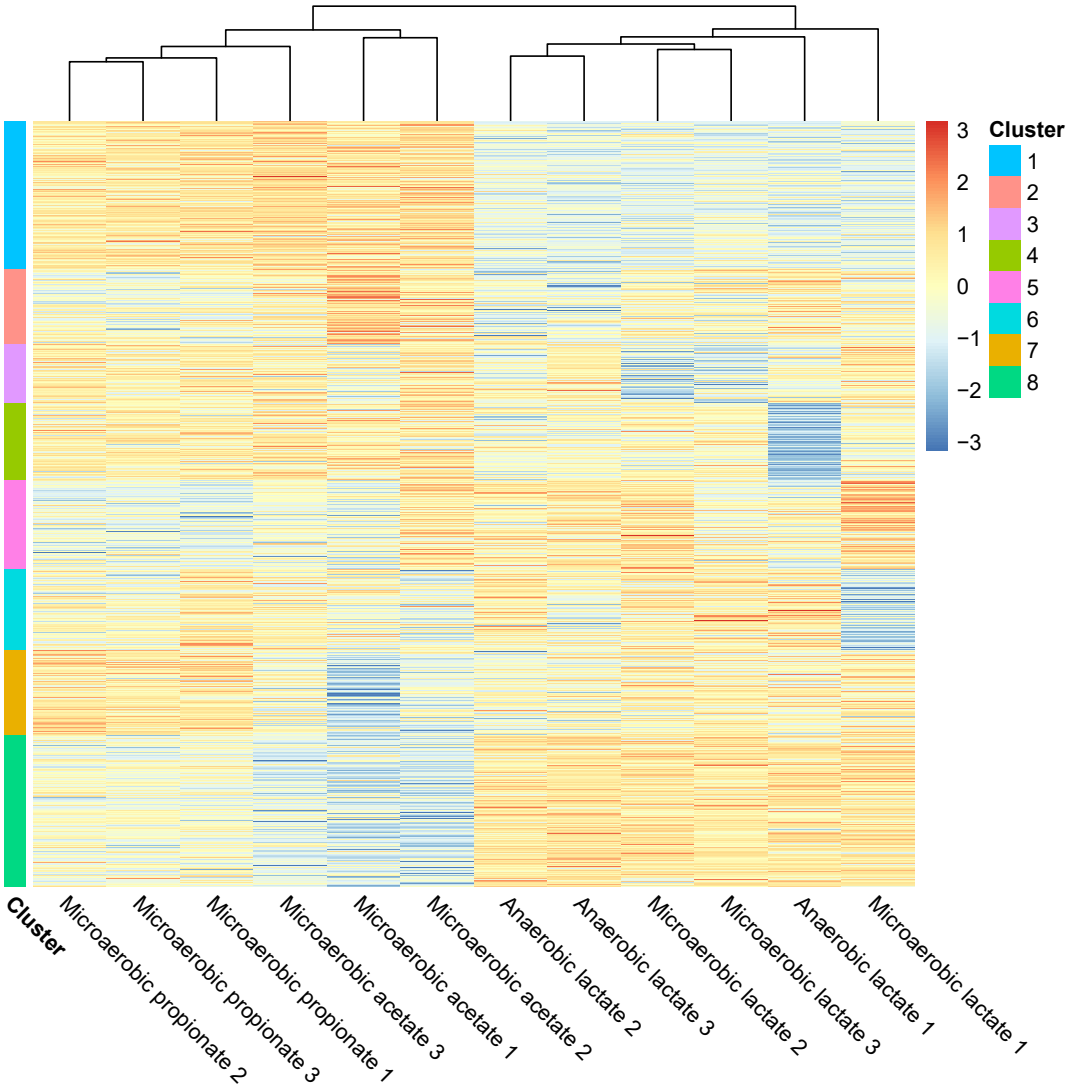

Figure 2.5: Hierarchical clustering of proteomes from cells growing in anaerobic or microaerobic conditions on lactate (anaerobic and microaerobic) and propionate and acetate (microaerobic). K-means clustering with k=8 was applied to cluster detected proteins into protein clusters with similar abundance patterns across each sample. Protein clusters can be found in supplementary file 3.

The modified TCA-cycle suggested by Beck and Schink (1995), menaquinone biosynthesis proteins, heme biosynthesis proteins and cytochrome bd-oxidase subunit I (*cydA*) were all found to be expressed in anaerobic and microaerobic conditions. The expression of cytochrome bd-oxidase remained stable in anaerobic and microaerobic conditions. Cytochrome bd is characterized by a high affinity for O_2 and is preferentially expressed at low O_2 tension (Giuffrè et al. 2014; Tseng et al. 1996) but is also expressed at similar levels in anaerobic conditions in several bacteria

(Govantes et al. 2000; Machado et al. 2006). Cytochrome bd-oxidase is clearly linked to conditions in which the high-affinity to oxygen (Baughn and Malamy 2004; D'mello et al. 1996) of cytochrome bd-oxidase becomes beneficiary for the scavenging of oxygen. The expression of cytochrome-bd oxidase in *P. freudenreichii* even in anaerobic conditions suggests that *P. freudenreichii* is prepared for the availability of O_2. In line with our results, in *Cutibacterium acnes* the whole respiratory chain was also expressed in anaerobic conditions (Brzuszkiewicz et al. 2011). Proteins needed for respiration processes are thus abundant in anaerobic conditions in the absence of additional external electron acceptors. We hypothesize this behaviour reflects adaptation to an environment in which variable but low supply of oxygen prevails. In such an environment, possession of an electron transport chain with high affinity for oxygen is beneficial, while such an enzyme system does not need to cope with high concentrations of molecular oxygen. This hypothesis fits the observations that oxygen is toxic at high concentrations (Vries et al. 1972), but at low rates can improve biomass production (this study and Pritchard et al. (1977)).

Microaerobic propionate and acetate consuming cells show distinct proteomes compared to lactate grown cells

The proteins involved in the conversion of lactate to pyruvate, Wood-Werkman cycle, acetate production and TCA cycle were not found to be significantly different in abundance when comparing anaerobic and microaerobic conditions for cells growing on lactate. Hierarchical clustering revealed proteomes were highly similar between anaerobic and microaerobic lactate-grown cells (figure 2.5). Only 12 proteins were found to be differentially expressed, supporting the evidence for similar expression patterns amongst lactate-grown cells. This points towards fermentative behaviour whenever lactate is present, even in microaerobic conditions, which is confirmed by the production of propionate. Cells growing on propionate and acetate show distinct proteomes from lactate grown cells. This is reflected by the higher amount of significantly different expressed proteins compared to microaerobic lactate consuming cells (43 for propionate consuming cells and 70 for acetate consuming cells (figure 2.4)). Clear protein clusters with different expression patterns between

lactate-grown and propionate or acetate-grown cells were revealed using k-means clustering (cluster 1,5 and 8).

Aerobic respiration is upregulated in propionate and acetate consuming cells

Cluster 1 consist of proteins upregulated in microaerobic propionate and acetate-grown cells compared to anaerobic and microaerobic lactate-grown cells. Notably, the cluster contains proteins involved in pyruvate metabolism (pyruvate dehydrogenase, pyruvate ferredoxin oxidoreductase), as well as ferredoxin itself, indicating higher activity towards production of acetate (Marcellin et al. 2020). Several proteins in the TCA cycle were also grouped in cluster 1: Citrate (si)-synthase (*gltA1, gltA2*), citrate(isocitrate) hydro-lyase (*acnA*), succinate-semialdehyde dehydrogenase (*gabD*). This indicates further upregulation of proteins involved in the TCA cycle in cells growing on propionate and acetate. Cytochrome bd-oxidase subunit I and NAD(P)H:quinone oxidoreductase, which were found to be significantly different expressed (figure 2.4), were also present in this cluster, in line with further activation of aerobic electron transport. Cytochrome P450 was also present in this cluster. Cytochrome p450 is a b-type cytochrome (Munro and Lindsay 1996) containing heme, involved in oxidative metabolism of a wide variety of substrates (Omura 2005). NADH:flavin reductase was significantly more abundant in cells growing microaerobic on lactate compared to anaerobic conditions and was also significantly more abundant in acetate-grown cells compared to microaerobic lactate-grown cells. NADH:flavin reductase regenerates NAD^+ by reduction of flavins with NADH (Fontecave et al. 1987). Energy production and reoxidation of NADH depend greatly on flavin-dependent enzymes in aerobic conditions (Vorobjeva 1999). These findings point towards further upregulation of aerobic respiration in microaerobic conditions in propionate and acetate consuming cells.

Microaerobic conditions trigger oxidative stress response

Cells growing in microaerobic conditions showed upregulation of oxidative stress related proteins. FrnE, a chaperone protein from the DSBA oxidoreductase family

that protects proteins from oxidation during oxidative stress (Khairnar et al. 2013), was significantly upregulated in cells growing on lactate in microaerobic conditions compared to anaerobic conditions. NrdJ, a vitamin B_{12}-dependent ribonucleotide reductase involved in DNA repair in aerobic conditions was upregulated in the microaerobic lactate consumption phase compared to acetate and propionate consumption phase. Interestingly, in the acetate consumption phase several proteins which have been linked to bacterial oxidative stress response in various bacteria, glutathione S-transferase (*P. freudenreichii*) (Falentin et al. 2010), FeS cluster assembly protein SufB (*E. coli*) (Outten et al. 2003), and two component response transcriptional regulatory protein MprA (*Mycobacterium tuberculosis*) (He et al. 2006) were less abundant compared to microaerobic lactate grown cells. This indicates *P. freudenreichii* cells growing microaerobically on lactate experience higher oxidative stress levels compared to cells consuming acetate. Oxidative stress from radical formation largely depends on activity of electron transport chains and is highest in the exponential growth phase (González-Flecha and Demple 1995). The decreased abundance of oxidative stress response proteins may thus be a result of a decreased activity of electron transport chains due to the lower growth rates caused by the slow consumption rates of propionate and acetate. This is supported by cluster 8, consisting of proteins that were most abundant in anaerobic and microaerobic lactate-grown cells, average abundant in propionate-grown cells and least abundant in acetate-grown cells. Several proteins involved in heme (*hemA, hemY, hemK*) biosynthesis were found in this cluster, indicating a decreased biosynthesis of heme, which is needed for aerobic respiration. Interestingly, also proteins involved in vitamin B_{12} synthesis (*cobA, cbiF, cbiM, cbiN, cbiQ, cbiL, cbiX, cobU*) were found in this cluster, pointing towards lower demands for vitamin B_{12} in the acetate consumption phase, in line with the decrease of vitamin B_{12}-dependent NrdJ.

Lower growth rates on propionate and acetate are reflected by the proteome

The large difference in growth rates on lactate (either anaerobic or microaerobic) and propionate or acetate was reflected by proteomic clustering, which revealed

a cluster with proteins with higher abundance in lactate-grown cells, average abundance in propionate-grown cell and lower abundance in acetate-grown cells (cluster 5). The main proteins in this cluster were related to biosynthesis processes: the 30S and 50S ribosomal proteins, DNA/RNA polymerase, aminoacyl-tRNA ligases (protein synthesis) and elongation factors. This reflects the higher growth rates in cells growing on lactate, either anaerobic or microaerobic, compared to propionate and acetate. This was supported by significant downregulation of several proteins related to transcription (HrdD, RmlN) (Hansen et al. 2008), synthesis of purines, thymidylate and methionine (5-formyl tetrahydrofolate cyclo-ligase, (Hansen et al. 2008)), sulfur amino acid biosynthesis (sulfate adenylyltransferase, (Ullrich et al. 2001)) and active growth (methionine aminopeptidase, (Shapiro et al. 2011) and RtcB (RNA ligase, (Chakravarty et al. 2012)) in acetate consuming cells. Together, these results indicate that *P. freudenreichii* cells consuming acetate impair their DNA,RNA and nucleotide synthesis and activate systems to become dormant and persistent, a survival strategy activated by a range of microorganisms including *E. coli* (Lewis 2005).

Biological niche propionic acid bacteria

Natural habitats of bacteria often include transition zones between oxic and anoxic environments. When diffusion of oxygen is lower compared to the consumption of oxygen by the microbial community, a transition zone with O_2 concentrations between oxic and anoxic levels exists (Morris and Schmidt 2013). Such environments with microoxic zones include soil aggregates (Tiedje et al. 1984), sediments, the gastrointestinal tract of animals (Morris and Schmidt 2013) and the human gut (Albenberg et al. 2014). Bacteria thriving in these environments often contain cytochrome oxidases of the *bd*-type, which have high affinity for oxygen (Baughn and Malamy 2004; D'mello et al. 1996) and therefor are functional in microoxic environments (Morris and Schmidt 2013) by enabling aerobic metabolic flux at extremely low oxygen pressures (Puustinen et al. 1991). Propionic acid bacteria contain *bd*-type cytochromes and are commonly found in soil (Hayashi and Furusaka

1979), silage (Merry and Davies 1999), the rumen of animals (Bryant 1959) and human intestines (Albenberg et al. 2014), which contain microoxic zones. In the gut diffusion of oxygen from epithelial cells creates a low constant flux of oxygen which is consumed by the gut microbiota in the mucosa (Morris and Schmidt 2013). Modulation of the pO_2 of the gut increased the abundance of *Actinobacteria*, including *Propionibacterium*, showing that these microorganisms benefit from low concentrations of oxygen being present (Albenberg et al. 2014). In our chemostat reactor the optimum gas inflow concentration contained a pO_2 of 16-32 mmHg, close to the baseline levels of pO_2 of 40 mmHg found in human intestinal tissues samples (Albenberg et al. 2014). Our results showed that at low concentrations of oxygen indeed propionic acid bacteria grow and benefit from oxygen. When oxygen is available, *P. freudenreichii* can utilise fermentation products using the electron transport chain. In this way, *P. freudenreichii* ensures maximum uptake of substrates. The enzymatic activities needed in anaerobic and microaerobic metabolism are largely overlapping, making it possible to consume propionate and acetate with limited further investments in terms of *de novo* protein synthesis. The ability to scavenge fermentation end products of other microorganisms in microbial communities, like lactate, propionate, acetate and propanediol (Saraoui et al. 2013) further links to microoxic zones, like the gut, rumen and soil as ecological niche for propionic acid bacteria. At lower pH regions in the gastrointestinal tract, like the caecum with a pH of 5.7 (Fallingborg 1999), passive diffusion of undissociated acids into the cell may occur. However, in more neutral regions of the gastrointestinal tract (pH up to 7.4 in the terminal ileum (Fallingborg 1999)) or in the rumen (pH up to 6.5 (Argyle and Baldwin 1988)) *P. freudenreichii* would greatly benefit from the presence of active propionate and/or acetate transporters. The ability to actively import these compounds using specific transporters instead of relying on passive diffusion needs to be investigated for *P. freudenreichii*.

Conclusion

Here we have shown propionic acid bacteria can greatly benefit energetically from aerobic respiration via electron transport chain with oxygen as terminal acceptor. We have shown that under low levels of oxygen supply, *P. freudenreichii* deploys fermentative behaviour on lactate via the Wood-Werkman cycle, and is able to subsequently oxidise its fermentation products propionate and acetate in a preferred order, pointing to an extension of its metabolic repertoire. The switch from the production of propionate to consumption of propionate is a phenomenon we propose to coin as the 'propionate switch' in analogy to 'acetate switch' described in *E. coli* (Wolfe 2005).

The ability of *P. freudenreichii* to utilise fermentation end products secreted by other microbes, next to lactate, now also propionate and acetate, in combination with a functional respiratory chain containing a high O_2 affinity cytochrome bd-complex, points towards a niche occupation in microoxic environments like the rumen and human intestine. The expression of all redox proteins, electron carriers and electron transport chain proteins in anaerobic conditions further support the hypothesis of the ecological niche of *P. freudenreichii*. The impact of (an)aerobic electron transfer chains on propionic acid bacteria ecology and colonisation of various ecosystems with low supply of oxygen requires further studies.

Experimental procedures

Strains and media

Sixteen strains of *Propionibacteriaceae* spp. (6 strains *Propionibacterium freudenreichii*, 2 strains *Acidipropionibacterium thoenii*, 5 strains *A. jensenii* and 3 strains *A. acidipropionici*) isolated from different dairy sources were obtained from the public culture collections DSMZ or BCCM (see supplementary file 1). All strains were initially cultivated using yeast extract lactate (YEL) containing per litre: 12.8 g L-lactic acid (16 g 80% L-lactic acid syrup, Sigma-Aldrich), 10 g tryptone (Oxoid), 5 g yeast extract (Oxoid), 5 g potassium dihydrogen phosphate. pH was adjusted

to 7.0 using 5 M NaOH prior to autoclaving at 121°C degrees for 15 min. Strains were stored in 30% (v/v) glycerol cryovials at -80°C degrees.

Culture conditions initial screening

Strains were streaked from -80°C stocks on YEL plates and incubated for 7 days at strict anaerobic conditions using anaerobic gas (gas mixture: 80% N_2, 10% CO_2, 10% H_2) and an oxygen catalyst at 30°C in anaerobic jars. For aerobic incubation single colonies were inoculated in 10 mL YEL in 100 mL shake flasks (± 1 cm culture layer thickness) and incubated at 30°C static and shaking (120 RPM) for 5 days. For anaerobic incubation single colonies were inoculated in 10 mL YEL in 50 mL Greiner tubes. Anaerobic samples were incubated at 30°C for 5 days in anaerobic jars at strict anaerobic conditions using anaerobic gas (gas mixture: 80% N_2, 10% CO_2, 10% H_2) and an oxygen catalyst at 30°C in anaerobic jars. The optical density was measured at 600 nm (OD_{600}).

Long term batch cultivation

A single colony of *P. freudenreichii* subsp. *freudenreichii* DSM 20271 was inoculated in 10 mL YEL and incubated at 30°C anaerobically for 2 days, after which 1% (v/v) was inoculated into bioreactors with a working volume of 500 mL (Multifors, Infors HT, Switzerland). The stirring speed was set at 300*rpm, the temperature was kept constant at 30°C and the pH was controlled at 7.0 by automatic addition of 5 M NaOH and 0.5 M HCl. The gas mix containing N_2 gas and air was supplied through a sparger at the bottom of the fermenter using a mass flow controller premixing gas at set values at a rate of 0.1 L/min. Cultivations were followed for a period of 400 hours. 10 mL samples were taken at different intervals.

Biomass quantification

Biomass was quantified by measuring the cell dry weight (CDW) concentration. Briefly, weighed samples were passed through pre-weighted membrane filters with a pore size of 0.2 μm (Pall Corporation, Ann Arbor, MI, USA) by a vacuum filtration

unit. Residual cell material was washed using demi water. Filters were dried at 80°C for 2 days and weighed to determine the CDW concentration in g/kg culture. For yield calculations cell dry weight values were calculated from OD values using a second-order polynomial relation (Mastrigt, Abee, et al. 2018).

Analysis of extracellular metabolites

Lactate, acetate and propionate were quantified by High Performance Liquid Chromatography as described by Mastrigt, Mager, et al. (2018).

Proteome analysis

Proteomic sample preparation and analysis

P. freudenreichii cells grown in the long term batch cultivation were collected in 1 mL tubes and the cell pellet was frozen at -80°C. Samples were taken at 30 hours for anaerobic conditions. In microaerobic conditions samples were taken after 30 hours (lactate consumption phase), 5 or 7 days (propionate consumption phase) and between 13 and 15 days (acetate consumption phase). Samples were washed twice with 100 mM Tris-HCl (pH 8) and resuspended in 100 μl 100 mM Tris-HCl. Samples were lysed by sonication for 45 s twice while cooling 1 min on ice in-between. The protein content was determined using Pierce Coomassie protein assay and samples were diluted to 1 μg/μl using Tris-HCl buffer (pH 8). Samples were prepared according to the filter assisted sample preparation protocol (FASP) (Winiewski et al. 2009) with the following steps: reduction with 15 mM dithiothreitol, alkylation with 20 mM acrylamide, and digestion with sequencing grade trypsin overnight. Each prepared peptide sample was analyzed by injecting 5 μl into a nanoLC-MS/MS (Thermo nLC1000 connected to a Q Exactive HFX Orbitrap) as described previously (Lu et al. 2011). nLC-MSMS system quality was checked with PTXQC (Bielow et al. 2016) using the MaxQuant result files. LCMS data with all MS/MS spectra were analyzed with the MaxQuant quantitative proteomics software package (Cox et al. 2014) as described before (Smaczniak et al. 2012; Wendrich et al. 2017).

Proteome data filtering, statistics and analysis

A protein database with the protein sequence of *P. freudenreichii* DSM 20271 (ID:UP000032238) was downloaded from UniProt. Filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups file were performed with Perseus (Tyanova et al. 2016). Reverse hits and contaminants were filtered out. Protein groups were filtered to contain minimally two peptides for protein identification of which at least one is unique and at least one is unmodified. Also, each group required three valid values in at least one of the experimental groups. Volcano plots were prepared based on the Student's t-test difference between samples. Volcano plots were produced in Rstudio (Racine 2012) using EnhancedVolcano (Blighe et al. 2018). Proteins were considered to be significantly different amongst sample groups if $p<0.01$ and at least a 4-fold change difference was detected. A heatmap was constructed using Pheatmap (Kolde and Kolde 2018) in R-studio. LFQ intensity values were normalized using Z-scores (Jain et al. 2005), after which hierarchical clustering was performed amongst samples using complete-linkage clustering in Pheatmap. Protein expression pattern analysis and consequent clustering based on expression pattern was performed by K-means partitioning (MacQueen 1967) in Pheatmap using k=8, determined by the gap statistic method (Tibshirani et al. 2001) and the elbow method using factoextra (Kassambara and Mundt 2017) in Rstudio.

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Conflicts of interest

The authors declare no conflict of interest.

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3

Bacterial microcompartment-dependent 1,2-propanediol utilization of *Propionibacterium freudenreichii*

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Abstract

Bacterial microcompartments (BMCs) are proteinaceous prokaryotic organelles that enable the utilization of substrates such as 1,2-propanediol and ethanolamine. BMCs are mostly linked to the survival of particular pathogenic bacteria by providing a growth advantage through utilization of 1,2-propanediol and ethanolamine which are abundantly present in the human gut. Although a 1,2-propanediol utilization cluster was found in the probiotic bacterium *Propionibacterium freudenreichii,* BMC-mediated metabolism of 1,2-propanediol has not been demonstrated experimentally in *P. freudenreichii*. In this study we show that *Propionibacterium freudenreichii* DSM 20271 metabolizes 1,2-propanediol in anaerobic conditions to propionate and 1-propanol. Furthermore, 1,2-propanediol induced the formation of bacterial microcompartments, which were visualized by transmission electron microscopy and resembled BMCs found in other bacteria. Proteomic analysis of 1,2-propanediol grown cells compared to L-lactate grown cells showed significant upregulation of proteins involved in propanediol-utilization (*pdu*-cluster), DNA repair mechanisms and BMC shell proteins while proteins involved in oxidative phosphorylation were downregulated. 1,2-Propanediol utilizing cells actively produced vitamin B_{12} (cobalamin) in similar amounts as cells growing on L-lactate. The ability to metabolize 1,2-propanediol may have implications for human gut colonization and modulation, and can potentially aid in delivering propionate and vitamin B_{12} *in situ*.

Introduction

P. freudenreichii is a Gram-positive, non-spore forming bacterium which has been linked to several potential health promoting effects, such as reducing intestinal inflammation, immunomodulation, modulation of intestinal motility and absorption, reduction of pathogen adhesion and enhancement of bifidobacteria (reviewed by Cousin et al. (2011)). *P. freudenreichii* is able to cope with a large variety of stresses (oxidative, bile salt, temperature) (Falentin et al. 2010) and consequently has good survival capabilities in the upper intestinal tract (Huang and Adams 2004). Survival of *P. freudenreichii* in the intestinal environment is supported by expression of pathways involved in the metabolism of substrates present in the intestinal environment, such as propanediol (Saraoui et al. 2013).

Propanediol is a major end product from anaerobic degradation of rhamnose or fucose by the human intestinal microbiota and serves as an important carbon source for propanediol utilizing bacteria, which can metabolize 1,2-propanediol into propionate, generating ATP, and into 1-propanol for maintaining redox balance. The metabolism of 1,2-propanediol produces the toxic intermediate propionaldehyde (Sampson and Bobik 2008). Some bacteria can protect themselves from toxic intermediates by encapsulating the enzymatic processes in self-assembling proteinaceous organelles called bacterial microcompartments (BMCs) (Axen et al. 2014). BMCs are typically about 40–200 nm in diameter and are made of three types of shell proteins: hexamers, pseudohexamers, and pentamers (Kerfeld et al. 2018). Based on the highly conserved domain of shell proteins, Axen et al. (2014) predicted the presence of these organelles in 23 different bacterial phyla, including *Actinobacteria* which includes the species *P. freudenreichii*. However, no experimental evidence of bacterial microcompartment mediated utilization of 1,2-propanediol has been shown in *P. freudenreichii* and its role as carbon source is yet to be elucidated.

BMC-mediated catabolism of substrates involving a toxic aldehyde intermediate is driven by three core enzymes: aldehyde dehydrogenase, alcohol dehydrogenase and phosphotransacylase (Axen et al. 2014). The signature enzyme in the propanediol

utilization pathway is propanediol dehydratase, a vitamin B_{12}-dependent enzyme catalyzing the reaction of 1,2-propanediol to propionaldehyde (Axen et al. 2014). Vitamin B_{12} is produced in high quantities by *P. freudenreichii* (Burgess et al. 2009) and it also requires vitamin B_{12} as a cofactor for a key enzyme in its characteristic Wood-Werkman cycle. It is suggested that *Actinobacteria* such as *P. freudenreichii* obtained the propanediol utilization (*pdu*) cluster through horizontal gene transfer from *Clostridiales* (Ravcheev et al. 2019), in which acquisition may have been supported by the ability of *P. freudenreichii* to produce vitamin B_{12} *de-novo* (Roessner et al. 2002). BMCs are also found in some pathogenic bacteria, such as *Salmonella enterica, Enterococcus faecalis, Listeria monocytogenes,* pathogenic *Escherichia coli* and *Clostridium perfringens* (Kerfeld et al. 2018). BMC-mediated utilization of 1,2-propanediol increases competitive fitness of pathogens in the gut and consequently has been linked to virulence (Jakobson and Tullman-Ercek 2016). However, symbiotic relationships depending on 1,2-propanediol metabolism have also been shown for beneficial *Lactobacillus reuteri* and *Bifidobacterium breve* (Cheng et al. 2020). The ability to degrade 1,2-propanediol may have similar implications for the bifidogenic capacity reported for *P. freudenreichii* (Kaneko et al. 1994) and consequences for gut modulation and competition with pathogenic bacteria. Furthermore, the active production of vitamin B_{12} during metabolism of 1,2-propanediol has not been studied yet in *P. freudenreichii*.

In this study we present evidence for BMC-mediated anaerobic growth of *P. freudenreichii* on 1,2-propanediol, evidenced by substrate utilization, propionate and 1-propanol production, and vitamin B_{12} synthesis. Using transmission electron microscopy and proteomics, we confirmed the presence of BMCs, Pdu BMC shell proteins and enzymes in *pdu*-induced *P. freudenreichii*.

Materials and methods

Strains, Culture Conditions, and Growth Measurement

P. freudenreichii DSM20271 was grown anaerobically (Anoxomat modified atmosphere, MART; 10% CO_2, 5% H_2, 85% N_2) at 30°C in 50 mL tubes containing 40 mL complex media containing per liter: 10 g tryptone, 5 g yeast extract, 5 g KH_2PO_4 supplemented with 100 mM L-lactate or 1,2-propanediol. All media was set at pH 7.0 by addition of 5M NaOH and was filter sterilized through 0.2 μm filters into sterile flasks. OD_{600} measurements and extracellular metabolite samples were taken daily for a time period of 7 days.

Analysis of extracellular metabolites using High Performance Liquid Chromatography

Culture samples were taken at various time intervals and were analyzed for extracellular metabolites by high performance liquid chromatography. 1 mL culture was centrifuged at 17000 x g for 1 minute and the supernatant was collected. 0.5 mL supernatant was treated with 0.25 mL Carrez A and 0.25 mL Carrez B, vortexed and centrifuged at 17000 x g for 2 minutes. 200 μL supernatant was stored in HPLC vials at -20 °C upon analysis. HPLC was performed as described by Zeng et al. (2019). Quantification was performed by addition of a standard curve containing L-lactate, acetate, propionate, 1,2-propanediol and 1-propanol.

Transmission Electron Microscopy

P. freudenreichii cultures were grown anaerobically at 30°C in 100 mM L-lactate or 1,2-propanediol containing media. Samples were collected after 6 days of incubation (early stationary phase for 1,2-propanediol-grown cells). About 10 μg of dry cell biomass was fixed for 2 h in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). After rinsing in the same buffer, a post-fixation was done in 1% (w/v) OsO_4 for 1 h at room temperature. The samples were dehydrated using ethanol. The dehydrated cell pellets were then embedded in resin (Spurr HM20)

for 10 h at 70°C. Thin sections (<100 nm) of polymerized resin samples were obtained with microtomes. After staining with 2% (w/v) aqueous uranyl acetate, the samples were analyzed with a Jeol 1400 plus TEM with 120 kV setting as described by Zeng et al. (2019).

Vitamin B_{12} quantification

After 7 days of incubation the vitamin B_{12} (cobalamin) concentration was determined using a microbiological assay (Vitafast vitamin B_{12} kit, R-biopharm) for *P. freudenreichii* grown in 66 mM L-lactate and 49 mM 1,2-propanediol medium. Briefly, 1 mL of culture was disrupted by bead beating (lysing matrix B, mp-bio) 2x 1 min at 4.5 m/s with 1 minute on ice in between. Samples were centrifuged and diluted with water to appropriate concentrations for the test kit and were heat-extracted for 30 minutes at 95°C. The samples were cooled to room temperature and vitamin B_{12} detection was performed according to the manufacturer protocol.

Proteomics

P. freudenreichii cells were cultured in media supplemented with 100 mM L-lactate or 100 mM 1,2-propanediol for 7 days. Cell pellets were harvested by centrifugation of 1 mL of sample at 17000 x g for 1 min in table top centrifuges and cell pellets were frozen at -80°C. Samples were washed twice with 100 mM Tris (pH 8) and resuspended in 100 μL 100 mM Tris. Samples were lysed by sonication for 45 s twice while cooling 1 min on ice. Protein content was determined using Pierce Coomassie protein assay and samples were diluted to 1 μg/μL using Tris-HCl pH 8. Samples were prepared according to the filter assisted sample preparation protocol (FASP) (Winiewski et al. 2009) with the following steps: reduction with 15 mM dithiothreitol, alkylation with 20 mM acrylamide, and digestion with sequencing grade trypsin overnight. Each prepared peptide sample was analyzed by injecting 18 μl into a nanoLC-MS/MS (Thermo nLC1000 connected to a LTQ-Orbitrap XL) as described previously (Lu et al. 2011). LCMS data with all MS/MS spectra were

analyzed with the MaxQuant quantitative proteomics software package (Cox et al. 2014) as described before (Smaczniak et al. 2012; Wendrich et al. 2017).

A protein database with the protein sequence of *P. freudenreichii* DSM 20271 (ID:UP000032238) was downloaded from UniProt. Filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups file were performed with Perseus (Tyanova et al. 2016). Reverse hits and contaminants were filtered out. Protein groups were filtered to contain minimally two peptides for protein identification of which at least one is unique and at least one is unmodified. Also, each group required three valid values in at least one of the two experimental groups. A volcano plot was prepared based on the Student t-test difference between samples. Volcano plots were produced in Rstudio using EnhancedVolcano (Blighe et al. 2018). Proteins were considered to be significantly different amongst sample if $p<0.05$ and 4-fold change difference was detected. KEGG gene set enrichment analysis was performed using Clusterprofiler (Yu et al. 2012) and 2-fold change difference amongst proteins. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD024700.

Predicting BMCs shell proteins

The Hidden Markov Models (HMMs) of two BMC shell protein domains listed as Pf00936 and Pf03319 were retrieved from the Pfam database to predict BMC shell proteins as described in Axen et al. (2014); Zeng et al. (2019). Shell proteins were predicted by a HMM search using the HMMER package and a local protein database of *P. freudenreichii* DSM 20271 genome (CP010341.1 (Deptula et al. 2017)). All hits with an e-value less than or equal to 1e-05 that correspond to a genomic record from Genbank, RefSeq, EMBL, or DDBJ databases were accepted as BMC shell protein homologs (Supplementary file 1).

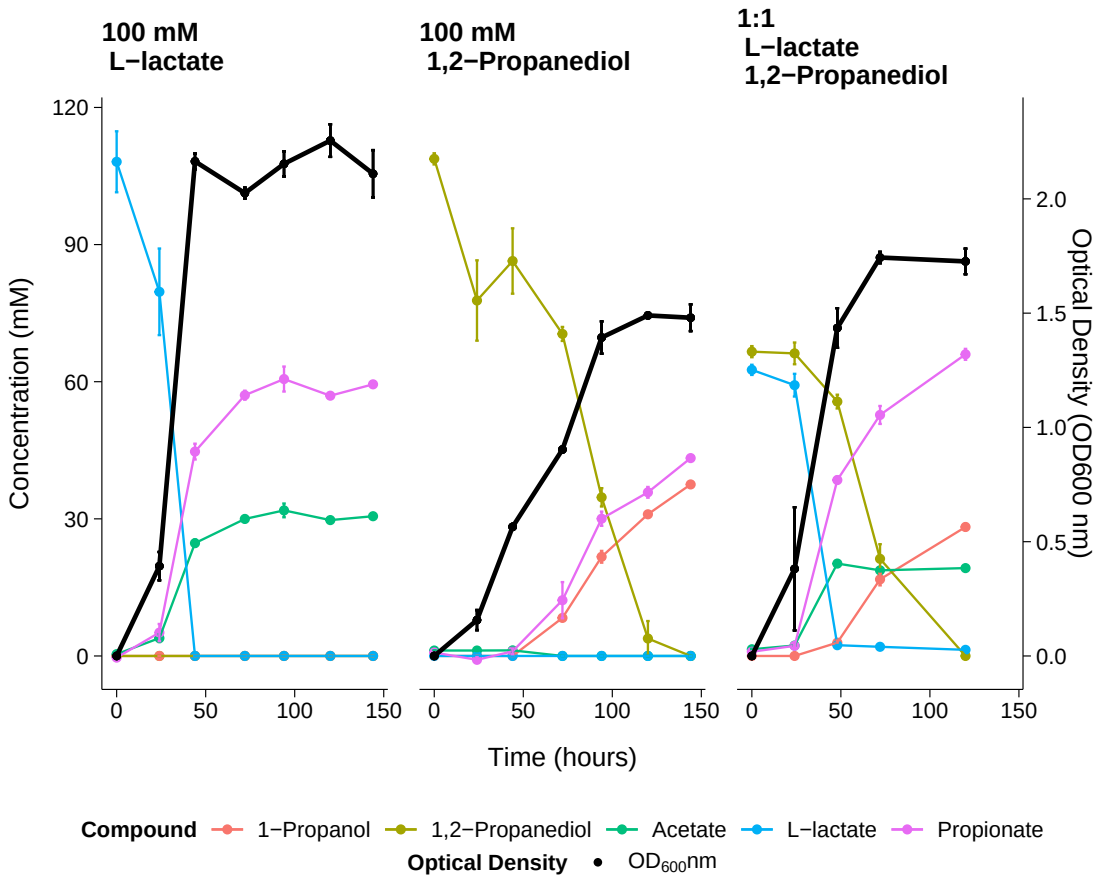

Figure 3.1: Substrate consumption, metabolite production and biomass formation of *P. freudenreichii* grown on 100 mM L-lactate, 100mM 1,2-PD and 1:1 1,2-propanediol:L-lactate for 6 days. Error bars = standard error of 3 biological replicates.

Results

Growth performance and metabolite production on 1,2-propanediol and L-lactate

Anaerobic growth of *P. freudenreichii* on 100 mM 1,2-propanediol was monitored for 6 days with daily sampling in triplicate. As a control, *P. freudenreichii* was cultured on 100 mM L-lactate. *P. freudenreichii* was able to completely metabolize 100 mM 1,2-propanediol during a period of 6 days (see Figure 3.1). This resulted in the production of 37.5 ± 0.1 mM 1-propanol and 43.2 ± 0.5 mM propionate. No production of acetate was observed, and with the current HPLC method used, approximately 28 mM of expected C_3 compounds is missing, conceivably volatile propionaldehyde. In the control cultures, 100 mM L-lactate was consumed within

2 days, resulting in 65.1 ± 0.1 mM propionate and 30.5 ± 0.1 mM acetate, close the expected molar ratio of 2:1 (Seeliger et al. 2002). Growth on 1,2-propanediol resulted in slower growth, with a stationary phase after 120 hours vs 48 hours in L-lactate. Our results clearly demonstrate that *P. freudenreichii* can grow on 1,2-propanediol, metabolizing it to propionate and 1-propanol.

To monitor metabolism of 1,2-propanediol in the presence of other carbon sources *P. freudenreichii* was grown in media containing both L-lactate and 1,2-propanediol. When NAD^+/NADH pools are shared between the cytosol and the BMC as discussed by Ferlez et al. (2019), the highest ATP yield from 1,2-propanediol in the presence of L-lactate can be obtained by co-fermenting 1,2-propanediol and L-lactate solely to propionate (see supplementary text 1). The expected ATP yield with L-lactate and 1,2-propanediol as mixed substrates is higher compared to cells growing solely on L-lactate (see supplementary text 1). We found production of 67.1 ± 1.5 mM propionate, 19.0 ± 1.0 mM of acetate and 28.3 ± 2.8 mM 1-propanol in cells growing on the mixed substrates. Assuming 1-propanol:propionate is produced in a 1:1 ratio from 1,2-propanediol and acetate:propionate is produced in a 1:2 ratio from L-lactate, 28 mM of the propionate originates from the 1,2-propanediol metabolism and 38 mM propionate originates from L-lactate metabolism. This matches the total found propionate of 67.1 ± 1.5 mM propionate in the samples. In mixed substrate conditions the amounts of total products formed indicate no apparent loss of C_3 compounds, which may indicate reduced loss of volatile propionaldehyde. Biomass formation with 1:1 L-lactate:1,2-propanediol-grown cells was found to be lower compared to 100 mM L-lactate-grown cells. These results indicate no apparent energetic benefit from mixed substrate conditions compared to mono substrate conditions, pointing towards independent pathways. Our results show mixed-substrate metabolism influences the total amount of short-chain fatty acid production, but pathway interactions are not apparent.

Vitamin B_{12} production

To demonstrate active vitamin B_{12} production under 1,2-propanediol utilizing conditions, *P. freudenreichii* was grown for 7 days in either yeast extract medium supplemented with 66 mM L-lactate or 49 mM 1,2-propanediol. Biomass formation, substrate utilization and vitamin B_{12} production by *P. freudenreichii* was monitored after 7 days of incubation. Incubation vessels were not opened in-between to prevent side effects by oxygen-dependent stimulation of vitamin B_{12} production, as has been shown for *P. freudenreichii* (Quesada-Chanto et al. 1998). Again, complete utilization of L-lactate and 1,2-propanediol was found (supplementary file 2).

Vitamin B_{12} was produced both when grown on L-lactate as on 1,2-propanediol (1.84 ± 0.11 μg/mmol L-lactate versus 2.19 ± 0.03 μg/mmol 1,2-propanediol). The specific vitamin B_{12} production when grown on L-lactate and on 1,2-propanediol was 270 ± 33 μg/g cells and 288 ± 27 μg/g cells, respectively (Figure 3.2). No significant difference was thus found for the production of vitamin B_{12} per g cells when growing on either L-lactate supplemented media or 1,2-propanediol supplemented media. In complex media, 1,2-propanediol as carbon source thus supports vitamin B_{12} production by *P. freudenreichii*.

Proteomics and electron microscopy of L-lactate and 1,2-propanediol grown cells

Cells growing in media supplemented with L-lactate (control) and with 1,2-propanediol were visualized using transmission electron microscopy (TEM). Thin sections of cells grown on 1,2-propanediol supplemented media clearly display cellular structures which were not found in the cells grown on L-lactate supplemented media (see Figure 3.3), and those structures resemble BMC structures found in other bacteria including *L. monocytogenes* (Zeng et al. 2019), *S. enterica* (Crowley et al. 2008) and *E.coli* (Toraya et al. 1979).

Proteome analysis revealed that BMC structural shell proteins (PduA, PduB, PduK, PduJ, PduN, PduM) and enzymes involved in 1,2-propanediol utilization (PduL, PduC, PduD, PduE, PduP, PduO, PduQ) were significantly more abundant

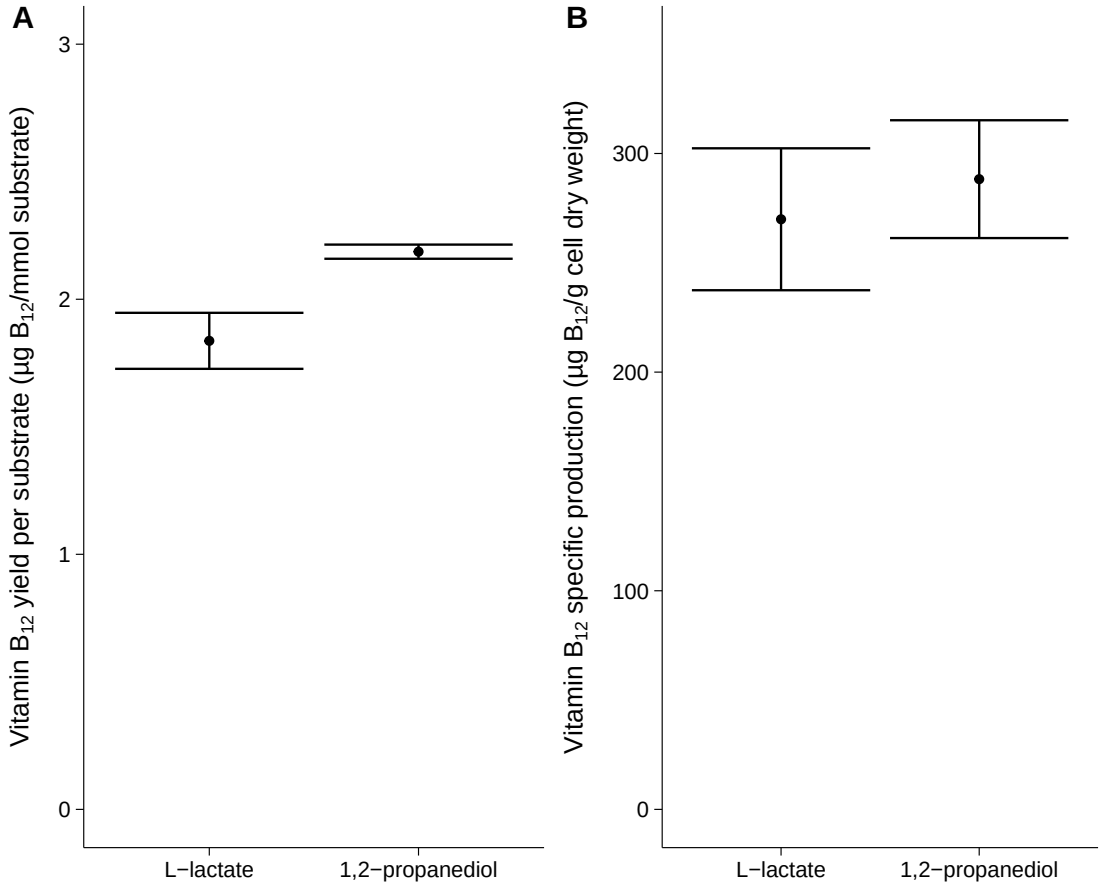

Figure 3.2: Vitamin B_{12} formation in cells grown on L-lactate or 1,2-propanediol. **A** vitamin B_{12} yield in μg/mmol substrate **B** Biomass specific vitamin B_{12} production in μg/g cell dry weight. Error bars = standard error of 3 biological replicates.

in cells grown with 1,2-propanediol as a substrate compared to cells grown on L-lactate (Figure 3.4). Lactate permease (LldP), succinate dehydrogenase subunits A, C1 (SdhA, SdhC1) were found to be significantly more abundant in the L-lactate-grown cells compared to 1,2-propanediol-grown cells, indicating a decrease of proteins in lactate-degradation pathways such as the Wood-Werkman cycle. Gene set enrichment analysis of KEGG pathways (see supplementary file 3) revealed upregulation of the propanediol degradation pathway in 1,2-propanediol grown cells compared to L-lactate grown cells (adjusted p-value <0.10). DNA repair mechanisms were also found to be activated in 1,2-propanediol-grown cells (adjusted p-value <0.10). In 1,2-propanediol-grown cells significant suppression of oxidative phosphorylation was found compared to L-lactate grown cells (adjusted p-value <0.01). The measured consumption of 1,2-propanediol, production of 1-propanol

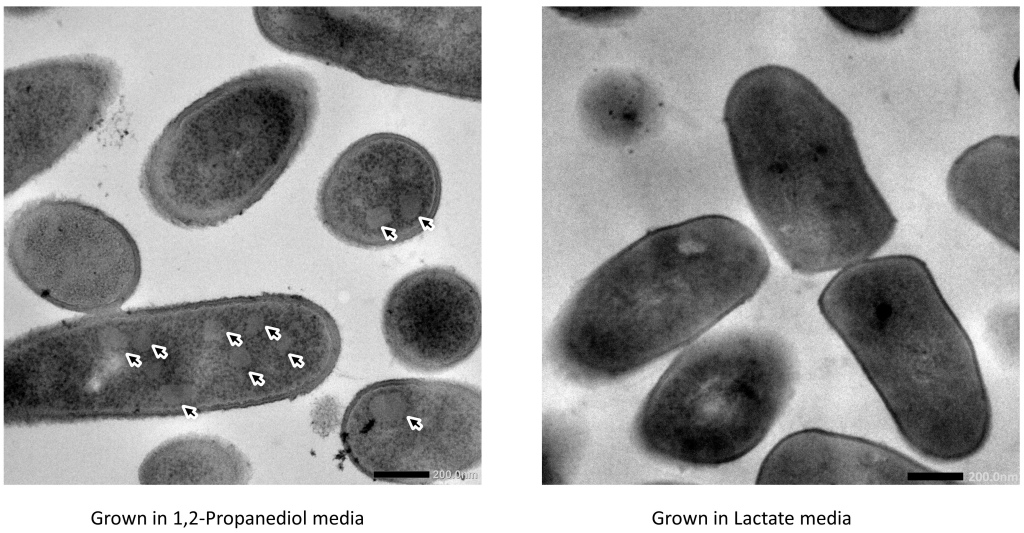

Figure 3.3: Visualization of bacterial microcompartments in propanediol media (left) and absence in lactate media (right).

and propionate, visualized cellular structures and strong upregulation of the *pdu* loci proteins provide evidence for activation of BMC-dependent 1,2-propanediol utilization in *P. freudenreichii* DSM 20271.

Overview of BMC-dependent 1,2-propanediol metabolism model in *P. freudenreichii*

Subsequent analysis of the *pdu* cluster in *P. freudenreichii* DSM 20271 identified two distant loci, with locus 1 starting from RM25_0852 to RM25_0857 and locus 2 starting from RM25_1258 to RM25_1273 (Figure 3.5A).

Locus 1 contains four genes which are *pocR* encoding a transcriptional regulator, *pduQ* encoding 1-propanol dehydrogenase, *pduV* with unknown function and *pduU* encoding BMC shell protein. Locus 2 carries 14 genes including 6 genes encoding BMC shell proteins and 8 genes encoding enzymes for the 1,2-propanediol degradation pathway and is not preceded by any known transcriptional regulators. Based on the structural studies of BMCs (Greening and Lithgow 2020; Sutter et al. 2017) and our understanding of 1,2-propanediol metabolism (Kerfeld et al. 2018; Sampson and Bobik 2008; Zeng et al. 2019), we propose the model of BMC-

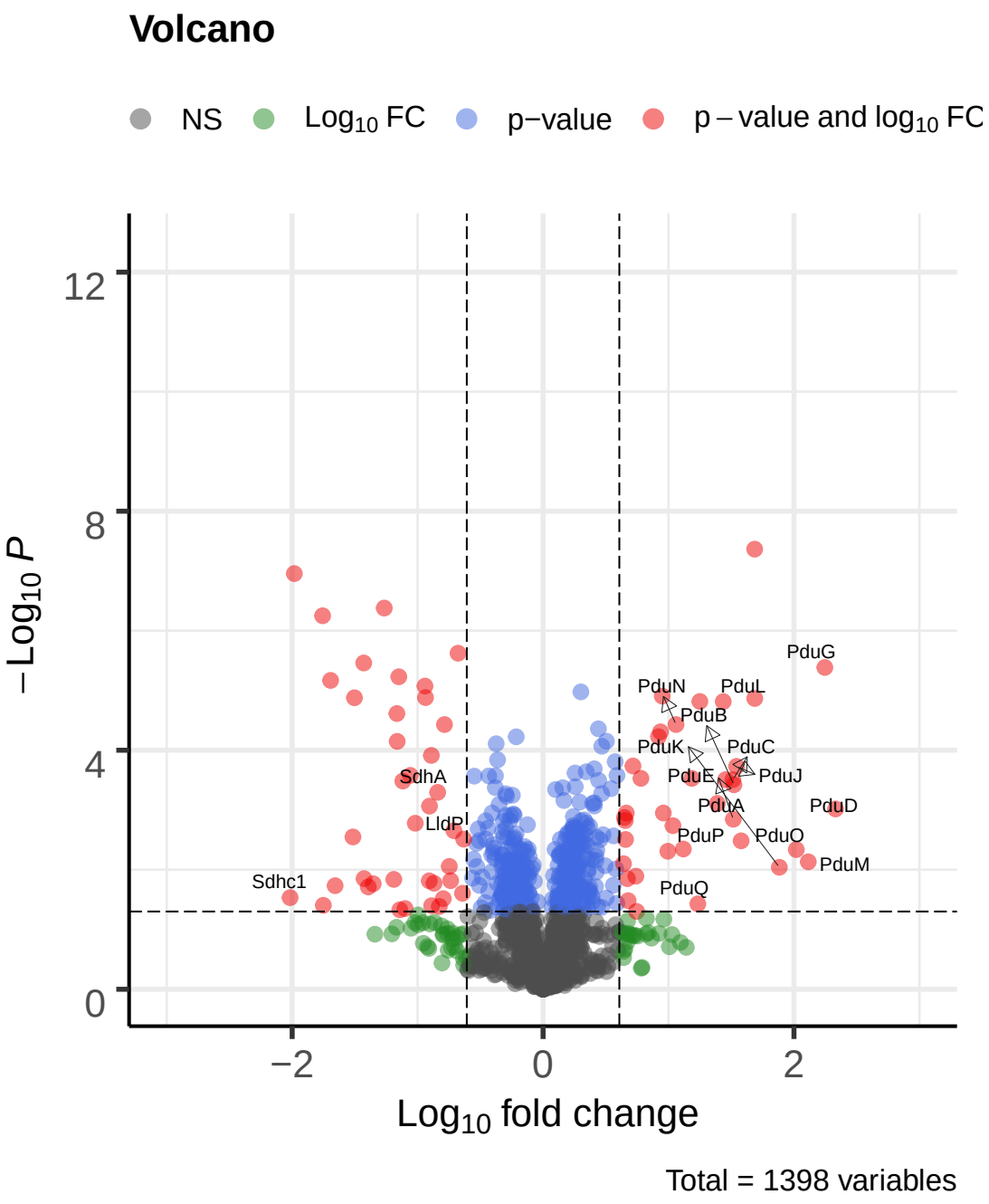

Figure 3.4: Volcano plot of proteomic analysis of cells grown in 1,2-propanediol media and L-lactate media. Positive $\log_{10}$ fold change indicate upregulation in 1,2-propanediol-grown cells, negative $\log_{10}$ fold change indicate down regulation in 1,2-propanediol-grown cells compared to lactate-grown cells. Red dots indicate proteins with $p<0.05$ and 4-fold difference expression. Blue dots indicate only $p<0.05$, green dots only 4-fold difference. Black dots indicate non-significant, non-differentially expressed proteins of 3 biological replicates

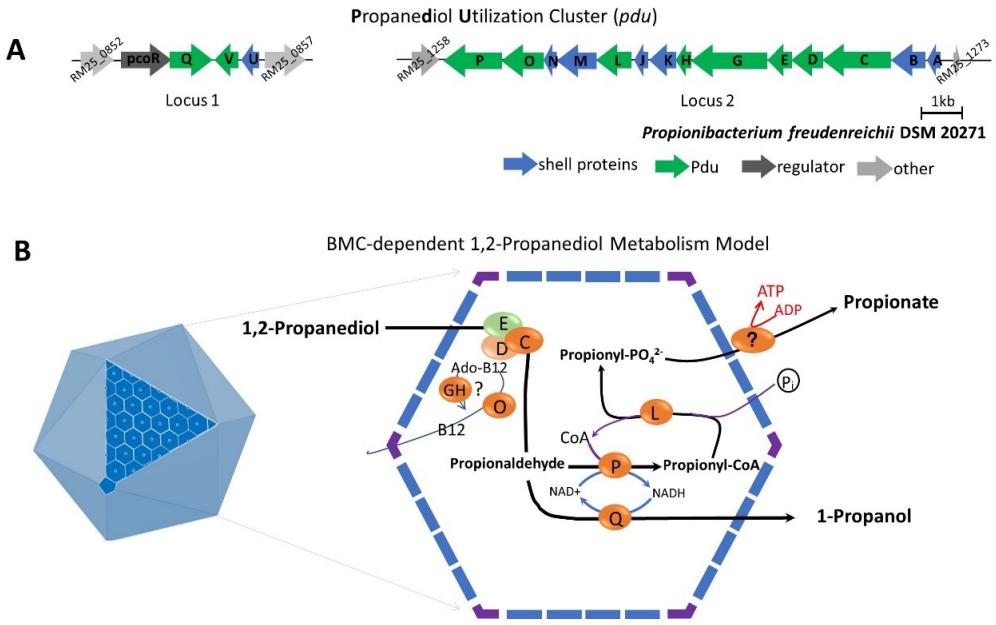

Figure 3.5: **A** Analysis of the *pdu* gene cluster (Details in supplementary file 1). Characters in green represent Pdu enzymes, in blue represent BMC shell proteins, in dark represent regulator and in grey represent unannotated proteins with gene ID. **B** Model of BMC-dependent 1,2-propanediol metabolism. In the left, the icosahedral diagram represent BMC with one surface showing the assembly of shell proteins. In the right, the metabolic pathway of 1,2-propanediol metabolism. PduCDE, B_{12}-dependent diol dehydratase; PduP, CoA-dependent propionaldehyde dehydrogenase; PduGH, diol dehydratase reactivase; PduO, corrinoid adenosyltransferase; PduL, phosphate propanoyltransferase; PduQ, propanol dehydrogenase. See text for details.

dependent 1,2-propanediol metabolism in *P. freudenreichii*, with the predicted BMC shell proteins PduA, PduB, PduK, PduJ, PduM, PduN and PduU constituting the self-organized icosahedral organelle (Greening and Lithgow 2020; Kerfeld et al. 2018). As illustrated in Figure 3.5B, the catabolism of 1,2-propanediol starts with the conversion of 1,2-propanediol to propionaldehyde by vitamin B_{12}-dependent diol dehydratase PduCDE. The toxic propionaldehyde is then converted to propionate by the enzyme CoA-dependent propionaldehyde dehydrogenase PduP, followed by action of phosphate propanoyltransferase PduL, and potential propionate kinase located in the cytoplasm, resulting in the end product propionate and the production of ATP. The other end product is produced following conversion of propionaldehyde by propanol dehydrogenase PduQ into 1-propanol. The diol dehydratase reactivase PduGH and corrinoid adenosyltransferase PduO are linked to the supply and

recycling of vitamin B_{12}.

Discussion

P. freudenreichii is commonly found in the rumen and colon of animals and in the human intestine (Bryant 1959). In these environments fucose and rhamnose are degraded to 1,2-propanediol by the present microbiota (Xue et al. 2008). We showed 1,2-propanediol can be further metabolized into propionate and 1-propanol by *P. freudenreichii*, thereby supporting anaerobic growth. Previously, a locus containing 15 genes involved in propanediol utilization was detected in *P. freudenreichii* (Falentin et al. 2010). *In vivo* gene expression analysis showed the *pdu* operon to be expressed in *P. freudenreichii* cells contained in the colon environment of a pig (Saraoui et al. 2013), pointing towards propanediol utilization in intestinal environments. Interestingly, in this study we identified the *pdu* cluster distributed in two different loci in *P. freudenreichii* DSM 20271. The presence of the *pdu* cluster seems to be species specific in propionic acid bacteria, as blasting the *pdu* cluster of *P. freudenreichii* against *Cutibacterium acnes, Acidipropionibacterium acidipropionici, A. thoenii* and *A. jensenii* did not result significant hits for key components in the cluster (see supplementary file 4). Presence of 1,2-propanediol in the medium induced expression of the two loci in *P. freudenreichii* DSM 20271 and resulted in BMC formation, 1,2-propanediol metabolism and consequently propionate and 1-propanol production. Our results show that expression of the *pdu* cluster from two different loci results in effective BMC formation and 1,2-propanediol metabolism. Indeed, BMC genes are split into two or more loci in 40% of the prokaryotic genomes containing BMCs (Abdul-Rahman et al. 2013). In *P. freudenreichii* DSM 20271, locus 1 is preceded by transcriptional activator *pocR*, which has been linked to activation of the *pdu* cluster in *Salmonella* (Chen et al. 1994). We did not find any annotated transcriptional regulator in the vicinity of locus 2. The presence of two different loci in many prokaryotic genomes suggests expression is controlled by additional regulators next to PocR. Indeed heterologous expression of BMCs uncoupled from their cognate transcriptional regulators has been reported previously

(Wilson 2021). The transcriptional regulation and activation of the two *pdu* loci and the role of *pocR* in *P. freudenreichii* requires further attention.

Upregulation of the *pdu* cluster and DNA repair mechanisms clearly indicated the crucial role of BMCs to protect *P. freudenreichii* from the toxic intermediate propionaldehyde produced in the degradation pathway. The ability to utilize substrates producing toxic intermediates upon degradation results in a competitive advantage to other gut microbiota (Jakobson and Tullman-Ercek 2016), as shown for ethanolamine utilization by *S. enterica* during intestinal inflammation (Thiennimitr et al. 2011). Interestingly, the presence of genes encoding metabolosomes for the utilization of ethanolamine and propanediol has been linked to pathogenicity and aids in anaerobic growth and colonization of foodborne pathogens *L. monocytogenes, C. perfringens* and *S. typhimurium* (De Weirdt et al. 2012; Korbel et al. 2005). It has been suggested for beneficial bacterium *L. reuteri* that competition for 1,2-propanediol could result in decreased proliferation of pathogens (Cheng et al. 2020). *P. freudenreichii* is considered to be non-pathogenic and has the generally recognized as safe (GRAS) status (Meile et al. 2008). Substrate competition for 1,2-propanediol by *P. freudenreichii* may thus exert similar effects as suggested for *L. reuteri* on growth of pathogenic bacteria. This is in line with reports that *P. freudenreichii* can decrease adhesion of pathogens to human intestinal mucus cells (Collado et al. 2007). Furthermore, *P. freudenreichii* stimulates the growth of beneficial bifidobacteria (HoJo et al. 2002; Satomi et al. 1999) thereby promoting a healthy gut microbiota. The role of BMC-mediated 1,2-propanediol utilization by *P. freudenreichii* and its importance for modulating gut microbiota composition, both by substrate competition and promoting other beneficial microbiota, requires further investigation.

Our study shows the potential of *P. freudenreichii* to substantially contribute to the production of propionate in the human gut. Next to 1,2-propanediol, also lactic acid (Macfarlane and Gibson 1997) is a major end-product of microbial fermentation, which consequently can also be fermented by *P. freudenreichii* and additionally contributes to the production of propionate. In mixed substrate

conditions both pathways remained active, albeit without apparent interaction based on metabolite formation. However, mixed substrate conditions decreased the loss of C_3, suggesting less loss of volatile propionaldehyde, thus potentially more efficient BMC assembly. The assembly of BMCs in mixed substrate condition requires further attention. Propionate is linked to many putative health effects (reviewed by Hosseini et al. (2011)) and can further stimulate bifidobacteria (Kaneko et al. 1994). The production of propionate from propanediol further supports the potential of *P. freudenreichii* as a probiotic.

The metabolism of 1,2-propanediol requires propanediol dehydratase, which is vitamin B_{12}-dependant (Axen et al. 2014). Vitamin B_{12} is actively produced by *P. freudenreichii* as it is essential cofactor in a key enzyme in the Wood-Werkman cycle, methylmalonyl-CoA mutase. Synthesis of vitamin B_{12} in *P. freudenreichii* follows the anaerobic pathway (Roessner et al. 2002) enabling production of vitamin B_{12} for anaerobic metabolism of lactate. Here we show vitamin B_{12} is produced in similar amounts in complex medium when cells metabolize 1,2-propanediol or L-lactate as carbon source. In *S. typhimurium pocR* mediated expression of vitamin B_{12} is induced in the presence of propanediol (Richter-Dahlfors et al. 1994). We also identified *pocR* upstream of *pdu* loci 1 in *P. freudenreichii* DSM 20271, but as discussed before its exact regulatory role in *P. freudenreichii* remains to be elucidated. Vitamin B_{12} production is also regulated by a vitamin B_{12} regulated riboswitch in *P. freudenreichii.* In *Propionibacterium* strain UT1 expression of vitamin B_{12} biosynthesis occurred at vitamin B_{12} concentrations of 750 μM, much higher compared to the vitamin B_{12} concentrations found in this study (~90 nM). The role of this riboswitch for vitamin B_{12} production during metabolism of 1,2-propanediol also remains to be elucidated. Based on our findings, we hypothesize that *P. freudenreichii* occupies a lactate and propanediol-rich niche in the gut environment. Symbiotic relationships have been shown for the production of vitamin B_{12} (Belzer et al. 2017; Sokolovskaya et al. 2020), which may be hard-wired in the vitamin B_{12} production of *P. freudenreichii.* As next to vitamin B_{12}-dependent lactate metabolism, BMC-mediated 1,2-propanediol metabolism

supports anaerobic growth of *P. freudenreichii*, thereby contributing to *in situ* vitamin B_{12} production in the gut.

This study presents evidence for BMC-mediated vitamin B_{12}-dependent utilization of 1,2-propanediol in *P. freudenreichii.* We have shown that 1,2-propanediol supports anaerobic growth of *P. freudenreichii*. It is conceivable that utilization of 1,2-propanediol could aid colonization of propionibacteria in the human gut to exert beneficial effects, such as delivering vitamin B_{12} and propionate *in situ*, but these aspects require further study.

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Author Contributions

AD and ZZ designed and performed the experiments. SB, AD and ZZ performed proteomics and analyzed data. ZZ, AD, ES, RN and TA analyzed data. ZZ, AD, ES and TA wrote the manuscript. All authors read, edited and approved the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

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4

Ethylene glycol is metabolized to ethanol and acetate and induces expression of bacterial microcompartments in *Propionibacterium freudenreichii*

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Abstract

Ethylene glycol (1,2-ethanediol) is a two-carbon dihydroxy alcohol that can be the end-product of various metabolic pathways including those involved in catabolism of plant-derived xylose and arabinose. Recently, evidence of bacterial microcompartment (BMC) mediated metabolism of ethylene glycol to ethanol and acetate was presented in *Acetobacterium woodii*. Here we show that *Propionibacterium freudenreichii* DSM 20271 is also able to convert ethylene glycol in anaerobic conditions to ethanol and acetate in almost equimolar amounts. The metabolism of ethylene glycol led to a moderate increase of biomass, indicating its metabolism is energetically favourable. A proteomic analysis revealed ethylene glycol induced expression of the *pdu*-cluster, which encodes a functional bacterial microcompartment involved in the degradation of 1,2-propanediol. Cross-examination of the proteomes of 1,2-propanediol and ethylene glycol grown cells revealed PDU BMC-expressing cells have elevated levels of DNA repair mechanism proteins and cysteine biosynthesis proteins. Based on our results we conclude ethylene glycol is metabolized in similar fashion as in *A. woodii* to acetate and ethanol with acetaldehyde as intermediate within the bacterial microcompartment. Our analysis of whole genome sequences of selected genomes of BMC-encoding microorganisms able to metabolize ethylene glycol through the same pathway as *A. woodii* and *P. freudenreichii* indicates a potentially broad-distributed dual role of the *pdu* operon in metabolism of ethylene glycol as well as 1,2-propanediol.

Introduction

Ethylene glycol (EG, 1,2-ethanediol) is a two-carbon dihydroxy alcohol that is used in various industrial applications, such as production of antifreeze agent and as a precursor for the production of poly(ethylene terephthalate) (PET). Although usually made through chemical synthesis, various (native) pathways can be found in prokaryotes and eukaryotes that produce EG from a variety of substrates such as xylose, arabinose, lyxose and serine (reviewed by Salusjärvi et al. (2019)).

EG can be converted in aerobic conditions through the glyoxylate pathway to glyoxylate which subsequently can be metabolized to acetyl-CoA (Child and Willetts 1978). However, also an alternative (anaerobic) pathway is described, in which a diol-dehydratase acts on EG to produce acetaldehyde (Wiegant and De Bont 1980) which subsequently is converted to either acetyl-CoA or ethanol (Trifunovi et al. 2016). Next, acetyl-CoA is converted to acetate by substrate-level phosphorylation yielding ATP. Hence, the ability to utilize EG under anaerobic conditions through this pathway is energetically beneficial. A drawback of this pathway however is the formation of acetaldehyde, which is toxic to bacterial cells (Cheng et al. 2008) and is quickly lost from cells due to volatility (Penrod and Roth 2006). Recently, evidence was provided for the wide-spread existence of proteinaceous organelles in bacteria, so called metabolosomes or bacterial microcompartments, that enable encapsulation of metabolic pathways that yield toxic intermediates (Cheng et al. 2008; Sutter et al. 2021). One of these microcompartments is the 1,2-propanediol utilization (PDU) microcompartment, which is involved in the metabolism of 1,2-propanediol (1,2-PD) via propionaldehyde to propionate and 1-propanol (Crowley et al. 2008; Zeng et al. 2019). Although the main substrate for the PDU microcompartment is 1,2-PD, its activity has been shown as well on glycerol in *Limosilactobacillus reuteri* (Sriramulu et al. 2008). Additionally, utilization of glycerol through a BMC-mediated pathway has been suggested for other lactic acid bacteria (Bourdichon et al. 2021) and has been proposed to potentially occur in other *pdu*-encoding organisms as well (Zeng et al. 2022). Furthermore, in *Acetobacterium woodii* metabolism of EG was

reported to induce expression of the *pdu* cluster (Trifunovi et al. 2016) which results in the formation of bacterial microcompartments (Chowdhury et al. 2020). PDU microcompartments may thus be implicated in metabolism of a variety of substrates.

Recently, evidence for 1,2-PD utilization mediated through the PDU microcompartment was shown for *Propionibacterium freudenreichii* (Dank, Zeng, et al. 2021). 1,2-PD was metabolized to propionate and 1-propanol, was found to support biomass formation and induced bacterial microcompartments. *P. freudenreichii* is a food-grade bacterium used for production of propionate, vitamin B12 and cheese and has recently come into attention as potential probiotic bacterium (reviewed by Cousin et al. (2011)). As *P. freudenreichii* also encodes a functional *pdu* cluster it is interesting to explore additional substrates able to induce these bacterial microcompartments, such as EG. Furthermore, elucidating additional roles of bacterial microcompartments in *P. freudenreichii* increases our understanding of survival of *P. freudenreichii* in specific ecosystems.

In this study we investigated the potential of EG to be metabolized through a bacterial-microcompartment mediated pathway. Anaerobic metabolism of EG by *P. freudenreichii* was monitored and was found to result in formation of acetate and ethanol. A proteomic analysis revealed expression of the PDU proteins for cells growing on EG. This study provides evidence that the *pdu* cluster in *P. freudenreichii* is involved in multiple enzymatic conversions, similar to what was found for the microcompartment in *A. woodii*. In combination with other research we discuss the versatility of the *pdu* cluster encoding for a metabolic pathway that is able to convert multiple substrates. Finally, we discuss our results in the context of fermentation of plant-derived substrates such as xylose and arabinose.

Methods

Strain and preculture conditions

Propionibacterium freudenreichii DSM 20271 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and routinely grown on yeast

extract lactate (YEL) consisting per liter of: 10 g tryptone, 5 g yeast extract, 5 g KH_2PO_4 and 16 g 80% L-Lactate syrup (Sigma Aldrich) and 15 g bacteriological agar for plates. Cell cultures were grown for 3 days in liquid media in anaerobic conditions (anoxomat modified atmosphere; 10% CO_2, 5% H_2 and 85% N_2) and maintained in 30% (v/v) glycerol stocks at -80 °C. Cells were precultured for each experiment by streaking *P. freudenreichii* on YEL agar and incubating at 30 °C in anaerobic conditions for 7 days. Single colonies were inoculated in medium with composition described below.

Culture Conditions, and Growth Measurement

P. freudenreichii DSM 20271 was grown in anaerobic jars in anaerobic conditions at 30 °C in 15 mL tubes containing 10 mL complex media containing per liter: 10 g tryptone, 5 g yeast extract and 5 g KH_2PO_4. Media was supplemented with I) no supplement, II) lactate or III) ethylene glycol. All media was set at pH 7.0 by addition of 5 M NaOH and was filtered sterilized into sterile tubes through 0.2 μm sterile filters. OD_{600} measurements and extracellular metabolite samples were taken at various time intervals or only at end points (in separate experiments), for 3 independent biological replicates.

Analysis of organic acids

Culture samples were taken at various time intervals and were analysed for extracellular metabolites by high performance liquid chromatography by the methods of Dank, Zeng, et al. (2021). 1 mL culture was centrifuged at 17,000 x g for 1 min and the supernatant was collected. 0.5 mL supernatant was treated with 0.25 mL Carrez A and 0.25 mL Carrez B, vortexed and centrifuged at 17,000 x g for 2 min. 200 μL supernatant was stored in HPLC vials at -20°C upon analysis. 200 μL supernatant was injected on a UltiMate 3000 HPLC (Dionex Germany) equipped with an Aminex HPX-87H column (300 x 7.8 mm) with guard column (Biorad). 5 mM H_2SO_4 was used as mobile phase with a flow rate of 0.6 mL/min at a column temperature of 40 °C. Compounds were detected using a refractive index detector (RefractoMax 520).

Quantification was performed by addition of a standard curve containing L-lactate, acetate, propionate, succinate, ethylene glycol, acetaldehyde and ethanol.

Proteomics

Proteomic analysis was performed according to the methods of Dank, Mastrigt, et al. (2021). *P. freudenreichii* cells grown for 7 days on ethylene glycol (50 mM) or lactate (50 mM) were harvested by centrifugation of 1 mL of sample at 17,000 x g for 1 min in table top centrifuges and cell pellets were frozen at -80°C. Samples were washed twice with 100 mM Tris (pH 8) and resuspended in 100 μl 100 mM Tris. Samples were lysed by sonication for 45 s twice while cooling 1 min on ice. Protein content was determined using Pierce Coomassie protein assay and samples were diluted to 0.5 μg/μl using Tris–HCl pH 8. Samples were prepared according to the filter assisted sample preparation protocol (FASP;(Winiewski et al. 2009)) with the following steps: reduction with 15 mM dithiothreitol, alkylation with 20 mM acrylamide, and digestion with sequencing grade trypsin overnight. Each prepared peptide sample was analyzed by injecting (5 μl) into a nanoLC-MS/MS (Thermo nLC1000 connected to an Orbitrap Exploris 480) as described previously (Lu et al., 2011). LCMS data with all MS/MS spectra were analyzed with the MaxQuant quantitative proteomics software package (Cox et al. 2014) as described before (Smaczniak et al. 2012; Wendrich et al. 2017) using protein database with the protein sequence of *P. freudenreichii* DSM 20271 (ID:UP000032238) downloaded from UniProt. Filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups file were performed with Perseus (Tyanova et al. 2016). Reverse hits and contaminants were filtered out. Protein groups were filtered to contain minimally two peptides for protein identification of which at least one is unique and at least one is unmodified. Proteomics was performed on 3 independent biological replicates. Also, each group required three valid values in at least one of the two experimental groups. A volcano plot was prepared based on the Student's t-test difference between samples (FDR=0.05). Volcano plots were produced in Rstudio

using EnhancedVolcano (Blighe, 2018). Proteins were considered to be significantly different amongst sample if $p < 0.05$ and 2-fold change difference was detected.

Analysis of proteomes of cells grown on 1,2-propanediol and ethylene glycol

Data for *P. freudenreichii* grown on 1,2-PD was retrieved from PRIDE repository using identifier PXD024700. Both EG and 1,2-PD were filtered for proteins with >2 fold expression compared to lactate. Using Uniparc identifiers unique and overlapping proteins were identified.

Results

Growth performance and metabolite production on EG

Anaerobic growth of *P. freudenreichii* on 50 mM EG was monitored for 7 days with daily sampling in triplicate. As a control, *P. freudenreichii* was cultured on the basal medium without additional carbon source. *P. freudenreichii* was able to grow on the basal medium without additional carbon sources, conceivably on amino acids, and on the medium supplemented with EG. Compared to the control condition a significant higher ($p < 0.05$, Student t-test) OD_{600} was found (figure 4.1A) for cells growing in medium supplemented with EG. In non-supplemented media an OD_{600} of 0.34 ± 0.01 was reached, whereas EG supplemented cultures reached an OD_{600} of 0.41 ± 0.01. This shows that EG supplementation results in higher biomass formation. EG can thus act as additional carbon source for *P. freudenreichii*.

Analysis of substrate consumption showed that 36.5 ± 1.3 mM EG remained after incubation for 7 days (see figure 4.1B) from the initial content of 49.8 ± 0.2 mM EG, meaning 13.3 ± 1.3 mM was metabolized (26,7% of EG). Acetate (8.3 ± 0.8 mM), succinate (2.6 ± 0.1 mM), propionate (5.7 ± 0.2 mM) and ethanol (3.3 mM ± 0.3 mM) were found in media supplemented with EG. In the non-supplemented media acetate (4.1 ± 0.2 mM), succinate (2.6 ± 0.1mM) and propionate (4.8 ± 0.2 mM) were found, whereas ethanol was not detected. The addition of EG thus resulted in extra production of acetate (4.2 mM more detected) and ethanol (3.3

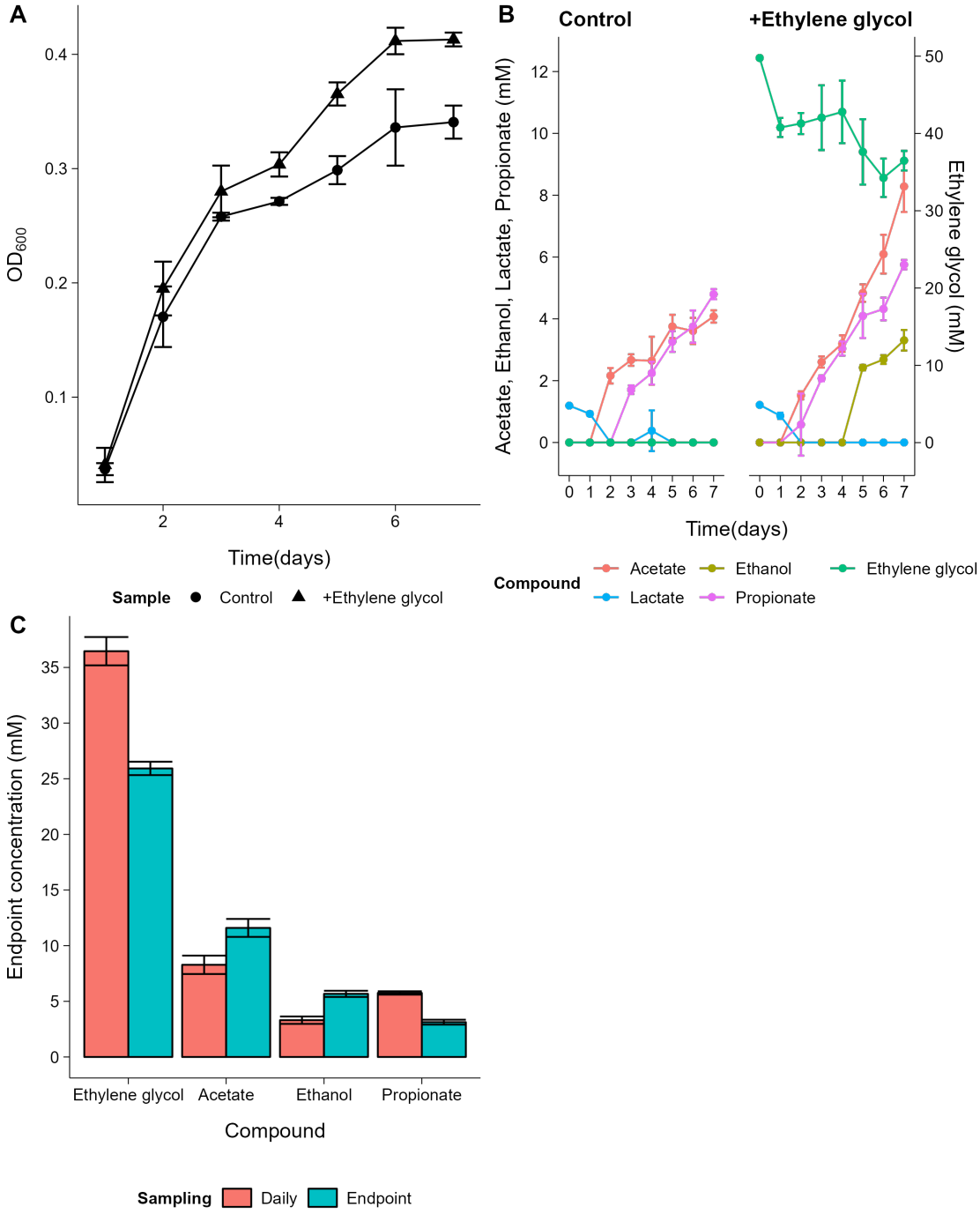

Figure 4.1: **(A)** Biomass production of *Propionibacterium freudenreichii* grown on basal medium (circles) and basal medium + 50 mM ethylene glycol (triangles). **(B)** Metabolite production (left y-axis) and ethylene glycol consumption (right y-axis) of *P. freudenreichii* grown on basal medium (left) and on basal medium + 50 mM ethylene glycol (right). **(C)** Substrate consumption and metabolite production at t=7 days of *P. freudenreichii* incubated for 7 days in basal medium and basal medium supplemented with 50 mM ethylene glycol sampled either daily or only at endpoint. Datapoints represent data of 3 independent biological replicates. Error bars represent standard deviation.

mM). Surprisingly, also a significant ($p<0.05$, Student t-test) higher amount of propionate was found. Assuming that both the acetate and ethanol formed in higher amounts originate from EG metabolism, a total of 7.5 mM of EG can be accounted for. This means 6.5 mM of expected C_2 compounds (approximately 49%) are missing. This might be explained by loss of volatile acetaldehyde. The loss of carbon is in line with previous findings of BMC dependent 1,2-PD utilization in *P. freudenreichii* (Dank, Zeng, et al. 2021), where a loss of 28% substrate (1,2-PD) derived products (propionate and propanol) is reported. The loss of C_2 compounds seems to be associated with initial stages of growth, as in day 1 approximately 9 mM EG is missing, whereas no ethanol or additional acetate production can be observed. However, acetaldehyde was not detected (detection limit: 0.5 mM) in culture supernatants. When additional EG is taken up again after 4 days (approximately 6.3 mM), the produced ethanol (3.3 mM) and acetate (3.7 mM), with an acetate:ethanol ratio of 1.1, indicates no loss of substrate.

To correct for any potential effect of oxygen influx during sampling, we decided to perform also end point measurements to quantify substrate utilization and product formation after incubation in strictly anaerobic conditions. Analysis of samples taken after 7 days of incubation showed a significant increase in substrate utilization, i.e., 24.0 mM EG was utilized (48,4% of EG compared to 26.7% utilized in daily sampling). The strict anaerobic conditions resulted in production of 11.6 $\pm$ 0.8 mM acetate, 3.1 $\pm$ 0.2 mM propionate and 5.7 $\pm$ 0.3 mM ethanol (see figure 4.1C). When correcting for the metabolites found in the basal medium, production of 6.9 $\pm$ 0.8 mM acetate and 5.7 $\pm$ 0.3 mM ethanol was observed, an acetate:ethanol ratio of 1.2. Similarly as in daily sampling a large proportion of expected C_2 was missing (47.8%). The found acetate:ethanol ratio is apparently not affected by the potential influx of oxygen while sampling daily. Again a higher concentration of propionate was found in the EG supplemented medium compared to the basal medium, supporting our earlier findings. However, the concentration of propionate was found to be much lower in strict anaerobic samples compared to samples that were sampled daily, suggesting small oxygen influx allowed utilization

of additional substrates from the basal medium such as amino acids. To avoid any potential effects of oxygen on protein expression patterns strict anaerobic samples were used for analysis of the proteome.

Model of BMC-dependent EG metabolism in *P. freudenreichii*

In *P. freudenreichii* two distant loci encode the *pdu* cluster. Locus 1 starts from RM25_0852 to RM25_0857 and locus 2 starts from RM25_1258 to RM25_1273. Locus 1 encodes *pocR*, a transcriptional regulator, *pduQ* encoding 1-propanol dehydrogenase, *pduV* with unknown function and *pduU* encoding BMC shell protein. Locus 2 encodes 14 genes including 6 BMC shell proteins (*pduABKJMN*) and 8 genes encoding enzymes for the 1,2-PD degradation pathway (*pduCDE* encodes 1,2-PD dehydratase, *pduP* encodes CoA-dependent propionaldehyde dehydrogenase, *pduL* encodes phosphate propanoyltransferase, *pduGH* (annotated as *DhaG*), diol dehydratase reactivase and *pduO* encodes corrinoid adenosyltransferase (Dank, Zeng, et al. 2021)). Notably, *pduT* a BMC-shell protein is missing from the genome of *P. freudenreichii*. PduT has been implicated in electron transport through the BMC shell (Crowley et al. 2010) and was found to be non-essential for BMC assembly and functioning in *S. enterica* in laboratory conditions (Cheng et al. 2011). No PduW homologue is found in the genome of *P. freudenreichii,* instead acetate/propionate kinase (RM25_0078, AckA) is encoded on a distinct locus not associated with any other Pdu proteins. In *A. woodii* five potential gene clusters encoding Pdu cluster proteins involved in the formation of BMCs were identified. Several substrates were able to induce expression of the cluster encoding *pduABKNT*; 1,2-PD, 2,3-butanediol, EG and ethanol of which 1,2-PD showed the highest induction indicating this compound to be the preferred substrate (Chowdhury et al. 2020). Hence the *pdu*-cluster is induced during the metabolism of various substrates. Interestingly, *A. woodii* encodes multiple PduU proteins, which was hypothesized to play a role in selective entry of specific metabolites in the BMC (Chowdhury et al. 2020). Combined with existing knowledge on BMC formation (Dank, Zeng, et al. 2021;

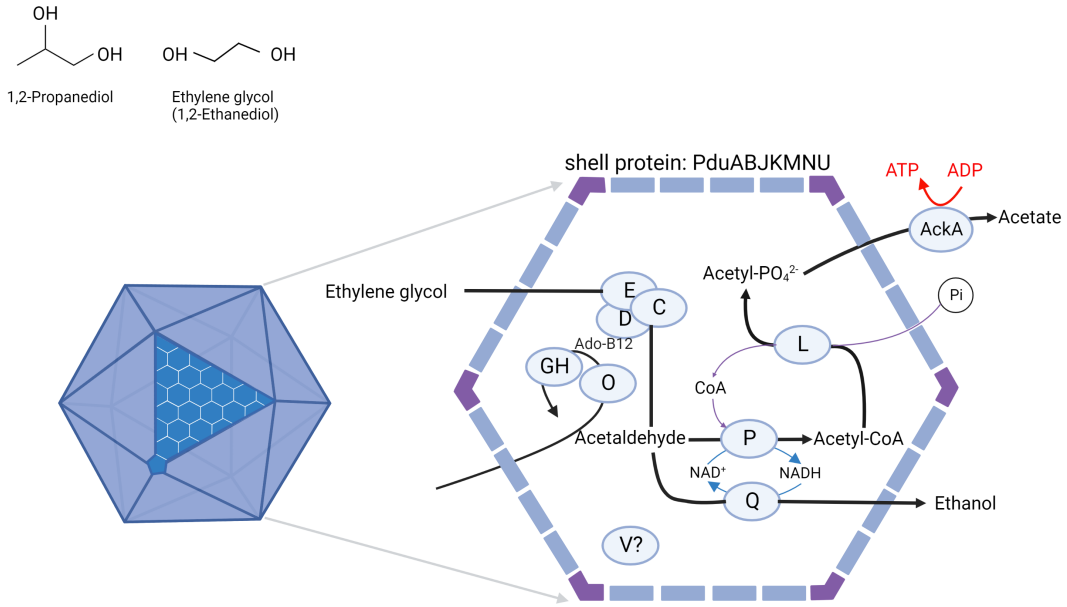

Figure 4.2: Proposed model of BMC-mediated ethylene glycol metabolism in *Propionibacterium freudenreichii*. The icosahedral diagram represents the BMC shell consisting of proteins PduABJKMNU. Inside the BMC: PduCDE, B12-dependent diol dehydratase; PduP, CoA-dependent propionaldehyde dehydrogenase; PduGH, diol dehydratase reactivase; PduO, corrinoid adenosyltransferase; PduL, phosphate propanoyltransferase; AckA, acetate kinase; PduQ, propanol dehydrogenase and PduV, of unknown function. See text for more details.

Sutter et al. 2021) and on the role of the *pdu*-cluster in metabolism of various substrates in *A. woodii*, we propose a model for BMC-mediated ethylene glycol metabolism in *P. freudenreichii* (figure 4.2).

Interestingly, in *P. freudenreichii* a hypothetical protein (RM25_1273) encoding 30 amino acids is predicted adjacent to *pduA*. When using protein BLAST for this hypothetical protein against the proteome of *A. woodii* it was found to be highly similar to small parts of conserved sequences in two PduU protein encoding genes (Awo_c28920 and Awo_c26570). The role and function of this small putative protein needs to be validated, but it is plausible that it is associated with a functionality within the BMC-shell in *P. freudenreichii*.

Proteomic analysis of EG-grown cells compared to lactate-grown cells

A proteomic analysis was performed for cells grown on media supplemented with lactate and cells grown on media supplemented with EG. Filtering of proteins upregulated at least 2-fold and with a p-value <0.05 revealed 70 proteins to be upregulated in EG-grown cells compared to lactate-grown cells. 13 of these proteins are encoded in the *pdu* cluster and were found to be highly induced in EG-grown cells (see figure 4.3 and Supplementary Table S1). We were able to identify major BMC shell proteins PduABJKMN. We were unable to detect PduU and PduV in our proteomes. Next to the shell proteins, we were also able to identify the complete 1,2-PD degradation pathway; 1,2-PD dehydratase (PduCDE), 1-propanol dehydrogenase (PduQ), CoA-dependent propionaldehyde dehydrogenase (PduP), phosphate propanoyltransferase (PduL) and acetate kinase (AckA). Furthermore Pdu proteins involved in B12 recycling were found; corrinoid adenosyltransferase (PduO) and diol dehydratase reactivase (PduGH, annotated as DhaG). Our results support the findings in *A. woodii* that EG is able to induce expression of the *pdu* cluster and is metabolized within the BMC (Chowdhury et al. 2020; Trifunovi et al. 2016).

In line with findings of Dank, Zeng, et al. (2021) we found a variety of proteins to be upregulated that are correlated to bacterial stress response (discussed below). Furthermore we found 2 proteins involved in the synthesis of pyridoxal (vitamin B_6), PdxT and PdxS, to be strongly upregulated (21 and 16-fold). PdxT and PdxS form an enzyme complex which synthesizes pyridoxal *de novo* from glutamine together with either ribulose 5-phosphate or ribose 5-phosphate and with either glyceraldehyde-3-phosphate or dihydroxyacetone phosphate (Richts et al. 2019). Vitamin B6 is a co-factor of many enzymatic activities amongst many involved in amino acid biosynthesis or catabolism. 21 different enzymes binding vitamin B6 (excluding pyridoxal biosynthesis genes) were identified by mass-spectrometry to be expressed in *P. freudenreichii* in the conditions used, of which cysteine synthase (RM25_1561) was found to be significantly upregulated (8-fold) compared

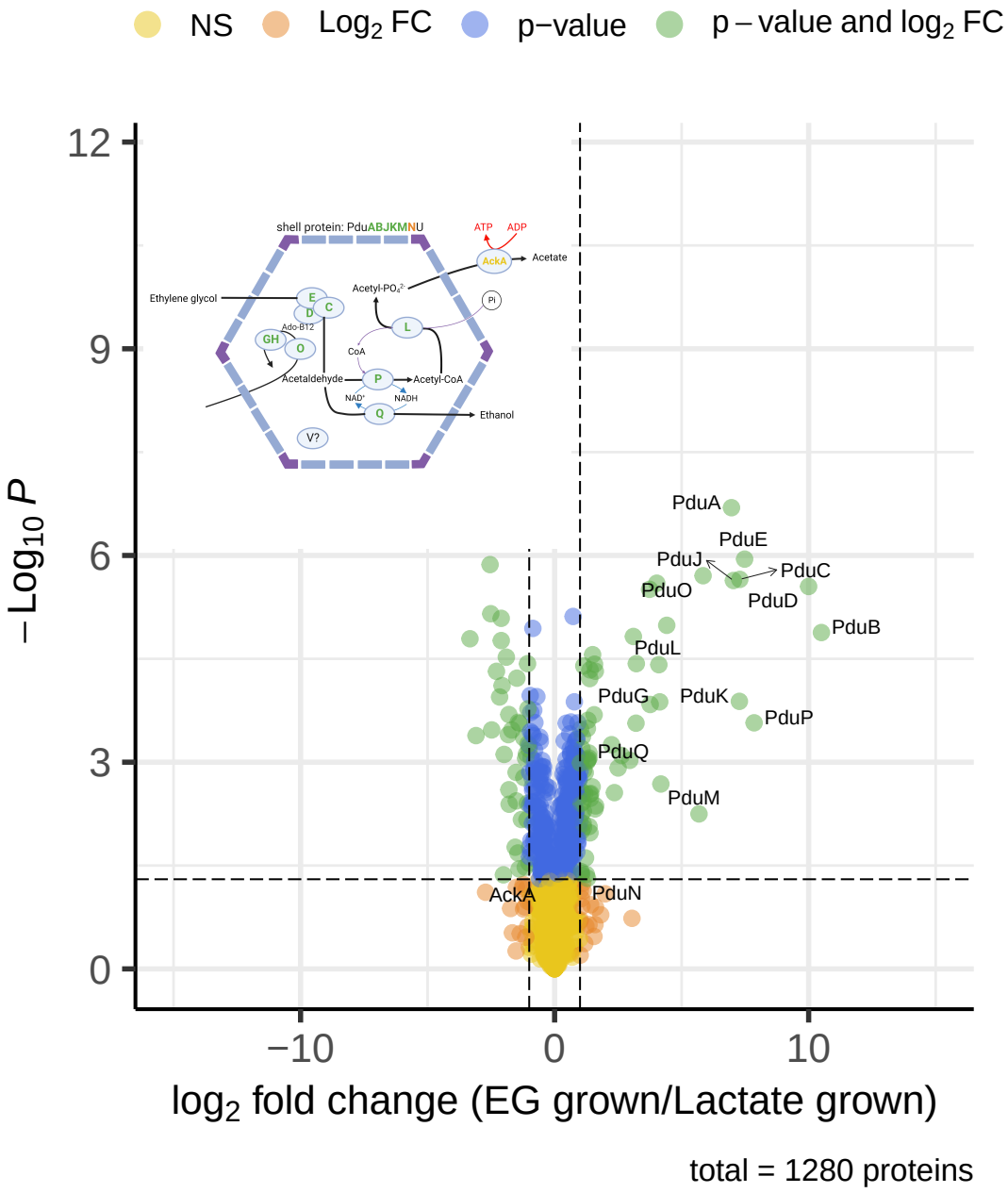

Figure 4.3: Volcano plot of proteomic analysis of cells grown in ethylene glycol media and L-lactate media. Positive log2 fold change indicate upregulation in ethylene glycol-grown cells, negative log2 fold-change indicate down regulation in ethylene glycol-grown cells compared to L-lactate grown cells. Data points consist of average of three individual biological replicates. Green dots indicate proteins with p below 0.05 and 2-fold change, blue dots indicate only p below 0.05, red dots only 2-fold change in expression, orange dots indicate non-significant, non-differentially expressed proteins. Arrows indicate upregulated PDU proteins. Top left-corner; Visualisation of protein expression levels in the proposed BMC-model.

to cells growing on lactate. Next to cysteine synthase, glutamate decarboxylase GadD and branched-chain aminotransferase IlvE were the only proteins that had both >2 fold expression and $p<0.05$ to require vitamin B6 as co-factor. The upregulated expression of pyridoxal producing proteins thus may result from a higher requirement of pyridoxal (derivatives) of upregulated proteins. Next to cysteine synthase, two other sulfur-metabolism related proteins were also highly upregulated: sulfite:ferredoxin reductase (RM25_2032, 9-fold) and sulfate adenylyltransferase (13-fold) (discussed below).

Identification of proteins upregulated in both 1,2-PD and EG grown cells

The switch from a metabolic mode revolving around lactate towards a BMC-mediated metabolism is likely to result in expression of additional proteins next to the *pdu* cluster. Since both 1,2-PD and EG induce expression of the *pdu*-cluster, these proteins should be upregulated in proteome samples of both cells compared to lactate-grown cells. To identify the proteins which are strongly associated with expression of the BMCs, we combined the proteomic data for both 1,2-PD (from Dank, Zeng, et al. (2021) and EG grown cells and filtered these datasets for proteins with less stringent criteria (at least a 2-fold expression increase compared to lactate-grown cells). For 1,2-PD cells this results in 164 proteins with >2-fold expression compared to lactate, for EG-grown cells in 87 proteins with >2-fold expression compared to lactate. Of these proteins, 46 proteins were expressed more than 2-fold in both 1,2-PD-grown and EG-grown cells (see figure 4.4 and Supplementary Table S2), with 13 of these proteins encoded within the *pdu* cluster(PduABCDEGJKLMOPQ).

Amongst the remaining shared proteins of 1,2-PD and EG-grown cells, multiple upregulated proteins have putative functions in DNA and RNA synthesis and repair processes. Genes encoding UvrD/REP helicase (RM25_1190) and ATP-dependent DNA helicase (RM25_1191) were upregulated. UvrD belongs to the UvrABC system responsible for nucleotide excision repair of which also RM25_1449 was upregulated (annotated as UvrABC system protein B). UvrD is a protein

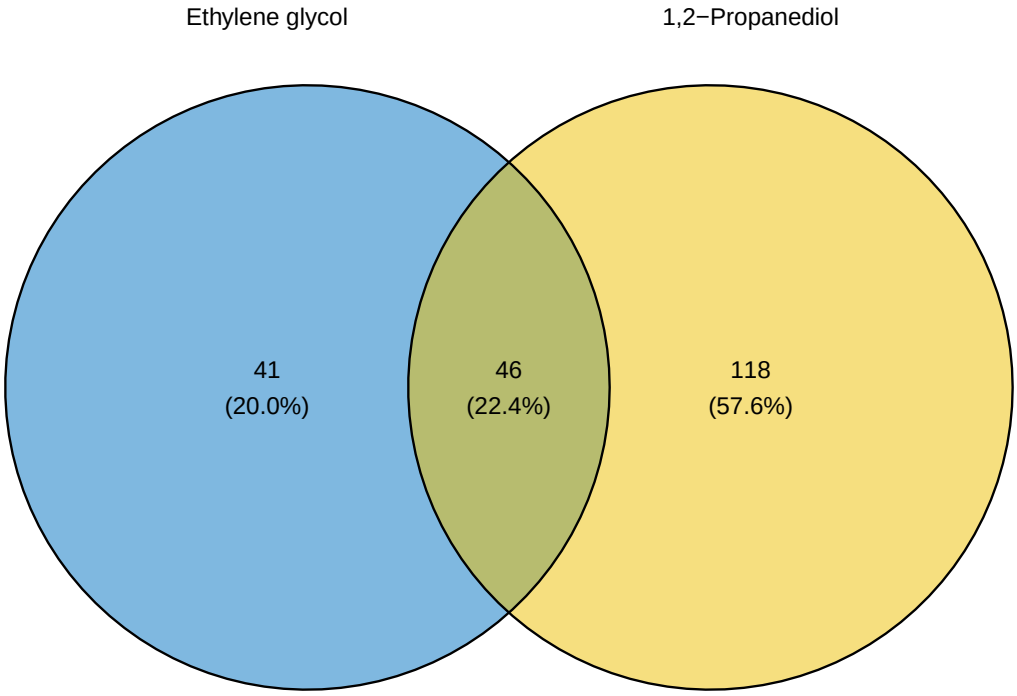

Figure 4.4: Venn diagram of proteins upregulated in ethylene glycol grown cells or 1,2-propanediol grown cells compared to L-lactate grown cells. Overlapping circles indicate proteins upregulated in both ethylene glycol as 1,2-propanediol grown cells compared to L-lactate grown cells. For a list of proteins see Supplementary Table S2.

possessing ATP-dependent DNA unwinding activities and important for repair, recombination, and replication of DNA (Kumura and Sekiguchi 1984). Also RM25_0677, formamidopyrimidine-DNA glycosylase, a base excision repair ligase (Boiteux et al. 1992) was upregulated. RadA (RM25_1921), a protein involved in genomic recombination and DNA repair (Beam et al. 2002; Burghout et al. 2007) was also more abundant in *pdu*-induced cells. Next to several DNA repair proteins, also RtcB (RM25_2257) was found to be upregulated. RtcB is a RNA ligase expressed to mitigate stress-induced RNA damage (Tanaka and Shuman 2011). Upregulation of these proteins indicate that although BMCs are expressed, cells still experience high levels of oxidative stress and cells need to invest into expression of additional DNA-repair proteins. Indeed, in *S. typhimurium* LT2 deletion of

important DNA repair protein PolA leads to the inability to grow on ethanolamine (acetaldehyde intermediate), and 1,2-PD (propionaldehyde intermediate) and showed increased sensitivity to both propionaldehyde and acetaldehyde (Rondon et al. 1995). This requirement is likely caused by low cytoplasmic levels of toxic intermediate that are not retained or are generated outside of the BMC by alternative alcohol dehydrogenases (Sauvageot et al. 2002), as shown by low levels of propionaldehyde in wild-type *S. enterica* (Sampson and Bobik 2008). Our results suggest the metabolism of 1,2-PD and EG through a BMC-mediated pathway requires additional expression of DNA and RNA repair proteins and thus agrees with previous findings (Dank, Zeng, et al. 2021; Rondon et al. 1995; Zeng et al. 2019). Interestingly, in 1,2-PD-grown cells overexpression of several stress defence proteins is more than 2-fold higher compared to EG grown cells (RM25_1190,RM25_1191, RM25_1921 and RM25_2257). This observation remains to be elucidated, although it is plausible that reduced metabolic flux due to lower enzymatic affinities results in smaller background pools of acetaldehyde compared to propionaldehyde, resulting in lower oxidative stress levels. However, also other explanations such as differences in boiling point between the aldehydes, potential to escape the BMC and differences in molecular structure and reactivity may explain this observation.

Just like in EG-grown cells, also 1,2-PD-grown cells upregulated PdxT and PdxS. It thus seems pyridoxal plays an important role during metabolism of BMC-inducing substrates in *P. freudenreichii*. Cells growing on either 1,2-PD and EG also upregulate AldA (RM25_1838) encoding a putative aldehyde dehydrogenase. Assuming this protein is not associated with the BMC in *P. freudenreichii* this would imply cells growing on BMC substrates also activate alternative options to 'capture' aldehydes formed outside of the BMC by promiscuous enzymes or formed by incomplete BMCs. Strong upregulation of various sulphur-related metabolic reactions is also observed in cells growing in 1,2-PD and EG. Cysteine synthase (RM25_1561) is highly upregulated, together with sulphite:ferredoxin reductase (RM25_2032) and sulphate adenylyltransferase (RM25_2034). This points towards upregulation of the assimilatory sulphate reduction pathway used

to convert inorganic sulphate to sulfide, which is further incorporated into carbon skeletons of amino acids as cysteine or homocysteine (Brunold 1993; Koprivova et al. 2001). This is further supported by upregulation of methionine transport (RM25_0298), which indicates either higher demand for methionine directly or indirectly in the form of sulphur-containing amino acid. In *Lactobacillus acidophilus* cysteine synthase is upregulated in response to oxidative stress from H_2O_2 (Calderini et al. 2017). Likewise in *Staphylococcus aureus* cysteine synthase mutants were more susceptible to H_2O_2 (Lithgow et al. 2004). Upregulation of genes encoding enzymes in the cysteine biosynthesis pathway in *P. freudenreichii* thus likely is a response to increased oxidative stress experienced during BMC-mediated metabolism. Cysteine likely plays a role in maintaining protein stability, as disulphide bonds formed between cysteine are important for protein folding and conformation (Kadokura et al. 2003). The upregulation of DsbA oxidoreductase (RM25_0775), a protein responsible for disulfide-bond formation and hence correct protein folding (Paxman et al. 2009), supports our hypothesis of higher cysteine requirements and points towards additional cell requirements in either *de novo* protein formation or salvaging of damaged proteins in BMC-induced cells.

Combining these results it can be concluded the BMC-mediated metabolism is associated with additional levels of oxidative stress, resulting in a necessity to upregulate DNA, RNA and protein repair processes and consequently pathways providing the building blocks for these processes.

Discussion

Propionibacterium freudenreichii encodes a functional *pdu* cluster that is activated during metabolism of 1,2-propanediol, resulting in formation of bacterial microcompartments and metabolism of 1,2-propanediol into propionate, ATP and 1-propanol (Dank, Zeng, et al. 2021). Here we show ethylene glycol is metabolized into acetate and ethanol by *P. freudenreichii*, supporting additional biomass formation. Presence of ethylene glycol in growth media resulted in high upregulation of the *pdu* cluster, supporting evidence of its involvement in degradation of ethylene glycol. Based

on our results, results of Chowdhury et al. (2020) in *A. woodii* and previously reported metabolic pathways of ethylene glycol metabolism mediated by PduCDE in other microbes (discussed below) we conclude that ethylene glycol is metabolized within the PDU BMC in *P. freudenreichii*.

Metabolic profile of *P. freudenreichii* metabolizing ethylene glycol

During culturing on ethylene glycol we observed biphasic growth for *P. freudenreichii*. After one day, a significant proportion of ethylene glycol (~9 mM) is consumed, whereas no product formation is observed and no further apparent metabolism takes place until day 4. Thereafter, more ethylene glycol is metabolized and formation of ethanol and (additional) acetate is observed, together with increased biomass formation. This behaviour was previously observed for 1,2-PD utilization, where in the initial days consumption of 1,2-PD is also not associated with accumulation of 1-propanol or additional propionate (Dank, Zeng, et al. 2021). Interestingly, discrepancy between 1,2-PD uptake and metabolite accumulation in initial stages is also reported for *Listeria monocytogenes* (Zeng et al. 2019). In *L. monocytogenes* this discrepancy also leads to loss of expected C_3 compounds. Likewise, in *Li. reuteri* a discrepancy between substrate uptake and product formation is also found for 1,2-PD utilization, but this seems to be related to delayed metabolite excretion as no apparent loss of C_3 is reported and propionate and 1-propanol are excreted whilst 1,2-PD is not detected anymore in the culture medium (Sriramulu et al. 2008). It therefore is conceivable that when cells encounter EG or 1,2-PD, they accumulate these compounds in the cytosol and expression of the PDU BMC is initiated. Apparent loss of carbon in initial growth stages would be explainable by incomplete BMC formation due to BMC assembly kinetics and hence metabolism of 1,2-PD or EG by expressed diol dehydratase outside of fully assembled BMCs, leading to loss of volatile aldehydes. This hypothesis is supported by the observation that incomplete or not fully assembled BMCs in mutant strains in *Salmonella enterica* lose the ability to retain aldehydes and that BMCs function as conserving

mechanism of volatiles (Sauvageot et al. 2002). However, this would not explain the delayed excretion of metabolites observed in *Li. reuteri*, hence also cytosol-BMC kinetics seem to play an important role. The boiling point of acetaldehyde (20°C) is considerably lower compared to propionaldehyde (48°C) (Smith and Bonner 1951), which means the potential to exit cells as volatile gas in the used experimental conditions is higher for acetaldehyde, potentially explaining the higher observed losses of volatile intermediate on EG compared to 1,2-PD. Next to the hypothesized role of BMCs for protection against aldehyde intermediates, for EG utilization (with acetaldehyde as intermediate) the role of intermediate retention thus seems more relevant compared to 1,2-PD utilization in the conditions used in our experiments. Interestingly, we did not detect (detection limit 0.5 mM) acetaldehyde in culture supernatants, which implies that intermediate loss either occurs largely directly to the gas-phase due to high volatility and/or part of the acetaldehyde is lost in chemical reactions with DNA and/or proteins, resulting in formation of DNA and/or protein adducts (Heymann et al. 2018). Formation of such adducts by aldehyde interactions is supported by our findings of upregulated proteins involved in DNA repair and protein repair and stabilization. Upregulation of oxidative stress response is also reported in other studies (Dank, Zeng, et al. 2021; Zeng et al. 2019) and thus seems to be a general response to BMC-mediated metabolism with aldehyde intermediates. Although no other known pathways of EG metabolism are encoded in *P. freudenreichii*, it cannot be excluded part of EG is metabolized via an alternative metabolic pathway that requires further study.

The acetate:ethanol molar ratio detected in our study is ~1.2 This implies either increased flux toward acetate production over ethanol or that part of the ethanol was not recovered. Studies in other bacteria reported possible impact of NAD^+/NADH recycling (Huseby and Roth 2013), as observed in *S. enterica* PduQ mutants (Cheng et al. 2012), or links with anaerobic electron transfer, as observed in *L. monocytogenes* (Zeng et al. 2021) and *S. enterica* (Price-Carter et al. 2001). However, we did not find evidence for such reactions in earlier studies on BMC dependent 1,2-PD utilization in *P. freudenreichii* (Dank, Zeng, et al. 2021), and

the only BMC-shell protein implicated with redox reactions across the BMC-shell (PduT) (Crowley et al. 2010), is absent in the genome of *P. freudenreichii*. An alternative hypothesis could be that ethanol partly evaporates from the culture media (Rodriguez et al. 1992). Our results also indicate that strict anaerobic conditions enhance ethylene glycol utilization. Oxygen has the potential to affect PDU BMC proteins, as reported for PduQ (Cheng et al. 2012) and diol dehydratase encoded in *S. enterica* (Bobik et al. 1999). One potential additional role of the BMC is hypothesized to shield redox-sensitive enzymes from oxygen (Kerfeld and Erbilgin 2015). Daily sampling may thus have resulted in partial Pdu enzyme inactivation, reducing the amount of EG that could be utilized.

Ethylene glycol grown cells showed significant upregulation of major shell proteins PduABJKM, diol dehydratase (PduCDE), 1-propanol dehydrogenase (PduQ), Coa-dependent propionaldehyde dehydrogenase (PduP), phosphate propanoyltransferase (PduL), corrinoid adenosyltransferase (PduO) and diol dehydratase reactivase (PduGH, annotated as DhaG). PduN was found to be more than 2-fold expressed, but did not meet the $p<0.05$ treshold. AckA was detected, but no significant difference in expression was found. PduU and PduV were not detected in our proteome analysis. PduU is one of the lesser abundant Pdu-proteins (Havemann and Bobik 2003) and is not required for correct BMC assembly (Crowley et al. 2008). The function of PduV remains unknown, although there is evidence it is implicated with spatial distribution of the BMCs within bacterial cells (Parsons et al. 2010). Theoretically it is possible BMC assembly occurs without expression of these genes, however we find it more plausible both proteins were not detected due to low transcription of the corresponding genes, as suggested earlier for PduT in *A. woodii,* which could not be detected using mass-spectrometry but was found to be expressed using gene expression analysis (Chowdhury et al. 2020). Hence, a similar approach is suggested for elucidating whether or not PduU and PduV are expressed in *P. freudenreichii*. Knock-out studies could be performed to elucidate which roles these proteins have for assembly, localization and for instance substrate transport across the BMC-shell in *P. freudenreichii*.

The metabolism of EG by other BMC-containing organisms

Evidence of EG metabolism by a BMC-mediated pathway containing a diol dehydratase as key enzyme has first been presented in *A. woodii*. In our study we have shown EG is also metabolized by *P. freudenreichii* in a BMC-mediated PDU pathway. Hence, it is very plausible other microorganisms containing B12-dependent BMC-PDU clusters have the capacity to metabolize EG. Alternatively, in a similar fashion B12-independent glycyl radical enzyme (GRE) microcompartments mediating 1,2-PD utilization may also support the use of EG as a substrate. As to our knowledge, no other experimental studies on the metabolism of EG and its relation to BMCs are reported in literature, except for the studies on *A. woodii* and our study. Hence the question arises whether any (or a large fraction of) BMC dependent PDU-containing organisms such as *S. typhimurium, L. monocytogenes* or *Li. reuteri* would be able to metabolize EG by a BMC-mediated PDU pathway and this topic is overlooked up to now, or whether novel types of BMC-mediated EG utilization exists.

To address this problem we identified organisms encoding *pdu* operons and/or B12-dependent diol dehydratases based on whole-genome sequencing and we combined this with experimental evidence of these organisms utilizing EG and 1,2-PD through the same pathway as *A. woodii* and *P. freudenreichii*, which have experimentally validated BMC-mediated EG metabolism. In *Levilactobacillus brevis* the diol dehydratase encoded by *pduCDE* was purified and showed activity on 1,2-PD, glycerol and EG (Sauvageot et al. 2002). Likewise, a B12-dependent diol dehydratase of *Secundilactobacillus collinoides* was isolated and showed similar activity on these substrates (Schütz and Radler 1984). However, these authors did not show product formation nor active metabolism/growth in these species. Growth and substrate utilization were shown for a variety of *Enterobacteriaceae* and *P. freudenreichii* on 1,2-PD and EG (Huseby and Roth 2013). *Citrobacter freundii, Citrobacter intermedium* and *Klebsiella pneumoniae* were able to grow on 1,2-PD and EG. *P. freudenreichii* DSM20271 was found to grow well on 1,2-PD but barely showed any biomass differences to the basal media after 3 days incubation on EG, in line with our findings that additional biomass formation

takes places after more than 3 days of incubation. The diol dehydratases encoded in these organisms formed propionaldehyde, showing the anaerobic pathway was followed. Based on their results, Toraya et al. (1980) conclude that the ability to ferment 1,2-PD is closely related to the metabolism of EG. However, any evidence that this was due to PDU BMC-mediated metabolism was not presented. For *K. pneumoniae* it was confirmed the diol dehydratase responsible for converting EG to acetaldehyde was B12-dependent, as the diol dehydratase of *K. pneumoniae* was found to convert EG to acetate and ethanol in equimolar amounts only in the presence of B12. When B12 was not present as cofactor, this diol dehydratase was unable to form acetate and ethanol from EG (Toraya et al. 1979), proving this enzyme indeed is B12-dependent and very likely the diol dehydratase encoded by *pduCDE* in the genome of *K. pneumoniae*.

Thus, evidence of anaerobic EG metabolism by (B12-dependent) diol dehydratases with acetaldehyde as intermediate does exist, although these studies originate from before the discovery of BMCs. *A. woodii, P. freudenreichii, C. freundii, Se. collinoides, Le. brevis* and *K. pneumoniae* all have in common that they contain the PDU BMC cluster (based on information from BMC-caller (Sutter and Kerfeld 2022) and (Chowdhury et al. 2020; Dank, Zeng, et al. 2021)). Based on the experimental validated activity on 1,2-PD and EG, reports of propionaldehyde/acetaldehyde intermediate formation and genomic information of these organism it is thus very likely EG is metabolized by the PDU BMC-mediated pathway.

Based on information from Sutter and Kerfeld (2022) at least three subtypes of PDU BMCs are implicated with metabolism of EG. *A. woodii* and *P. freudenreichii* contain subtype PDU1D, *C. freundii* and *K. pneumoniae* contain PDU1AB and *Se. collinoides* and *Le. brevis* contain PDU1C. It therefore seems unlikely that the activity of diol dehydratase on EG is restricted to one specific PDU BMC-subtype. Based on our knowledge of BMC induction by EG in *A. woodii* and *P. freudenreichii* and the evidence presented in other microorganisms we hypothesize that also other PDU BMC-encoding organisms would be able to ferment EG

using the BMC-dependent pathway in combination with expression of BMCs. Hence, experimental studies on PDU BMC-mediated EG metabolism using various PDU BMC-containing organisms such as *S. typhimurium*, *L. monocytogenes*, *Se. collinoides* and *Anaerobutyricum* spp. (previously known as *Eubacterium hallii*) are required. Interestingly, *C. freundii* and *K. pneumoniae* also encode EUT BMCs, which also have acetaldehyde as intermediate and acetate and ethanol as end-product. Although the signature enzyme ethanolamine ammonia-lyase likely does not display activity on EG, it cannot be excluded (part of the) EUT BMCs can be involved in EG metabolism, as the EUT shell and rest of the pathway may be better adapted for metabolism of EG due to the similarity in intermediates.

Ecological relevance

The ability to utilize EG through a fermentative BMC-mediated pathway may be coincidental due to its homology to 1,2-PD. Induction of the *pdu*-operon, permeability to the protein shell of the BMC and activity of 1,2-PD dehydratase would all need to be initiated by EG. The question thus arises to what extent EG is able to initiate all these processes and to what extent it can enter catalytic enzymatic sites. In *A. woodii* EG induces expression of the *pdu* operon, albeit at about 1/3rd of the expression found on 1,2-PD (Chowdhury et al. 2020). PduP converts propionaldehyde 3 times faster compared to acetaldehyde in *A. woodii* (Schuchmann et al. 2015; Trifunovi et al. 2016). Similarly, about a 3 times higher K_M value for EG versus 1,2-PD is reported for the PduCDE diol dehydratase purified from *Se. collinoides* and diol dehydratase purified from *Le. brevis* (Sauvageot et al. 2002; Schütz and Radler 1984). As a result of the lower expression and lower enzyme affinity for EG, *A. woodii* grows to much higher cell densities on 1,2-PD compared to EG (Chowdhury et al. 2020), behaviour which we also observed in *P. freudenreichii* grown on EG compared to *P. freudenreichii* grown on 1,2-PD (Dank, Zeng, et al. 2021). The difference reported between biomass formation on both substrates is less profound for *C. freundii, C. intermedium* and *K. pneumoniae*, but still a consensus for higher biomass formation on 1,2-PD is observed (Cheng

et al. 2012). Interestingly, in *T. glycolicus* a higher affinity for EG compared to 1,2-PD by diol dehydratase is reported, although this diol dehydratase is not B12-dependent (Hartmanis and Stadtman 1986) and also no *pdu*-cluster could be found in the genome of this organism, although some PDU shell proteins show significant hits when blasting against *P. freudenreichii pdu* cluster. It could be this organism encodes a GRE-type BMC with B12-independent diol dehydratase also acting on EG (Chowdhury et al. 2020; Sutter et al. 2021). Nonetheless, it seems diol dehydratase encoding organisms often have functionality towards EG which would support a claim for a duality in functionality evolved from underground promiscuous activity of PDU proteins (Khersonsky and Tawfik 2010).

Since the diol dehydratase in the PDU BMC is oxygen sensitive (Bobik et al. 1999) the ability to use this pathway for degradation of EG is most relevant in anoxic conditions or in environments containing only low amounts of oxygen. As described above, various pathways for the production of EG can be found in prokaryotes and eukaryotes. EG can be produced as end-product from a variety of substrates such as xylose, arabinose, lyxose and serine. Most pathways end up at glycoaldehyde which consequently is converted to glycolic acid or EG (reviewed by Salusjärvi et al. (2019)). However, many of these pathways are executed by aerobic bacteria. Native anaerobic pathways of interest are the Dahms pathway (Dahms 1974), in which D-xylose is degraded to xylonic acid and the D-ribulose-1P pathway in which D-arabinose is degraded to EG and glycolic acid (Pereira et al. 2016).

Xylose and arabinose are degradation products found after degradation of hemicellulose. Xylan is the most abundant plant polysaccharide after cellulose and the major polysaccharide in hemicellulose. Xylan consists mainly of D-xylose and arabinose monomers. Since xylans are a heteropolymeric substrate a variety of enzymatic activities is required to degrade it, including enzymes such as endoxylanases, β-xylosidases, α-l-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, and ferulic acid esterases (Dodd and Cann 2009; Dodd et al. 2010). With this variety of enzymatic functions xylans can be degraded to their respective monomers, such as D-xylose and L-arabinose (discussed in Dodd and

Cann (2009)). Xylan can be fermented by commensal bacteria in the human gut such as *Roseburia* and *Bacteroides* species and by *Prevotella* species in the bovine intestines (Chassard and BernalierDonadille 2006; Chassard et al. 2008; Chassard et al. 2007; Dodd et al. 2010; Salyers et al. 1982; Salyers et al. 1981). The gene cluster responsible for xylan degradation seems to be highly conserved amongst xylanolytic bacteria such as *Prevotella* (bovines) and *Bacteroides* (humans) spp. (Dodd et al. 2010). The xylanolytic activity results in the release of monomers, xylo-ologosaccharides and arabinoxylan-oligosaccharides which consequently can be utilized by other microbes such as bifidobacteria (reviewed in (Broekaert et al. 2011)). Xylanolytic activity inside the gut thus exerts health effects and consequently also results in the release of monomers such as D-xylose.

D-xylose itself can also be oxidized to xylonic acid. The oxidation of D-xylose can be catalysed by a non-specific glucose (aldolase) dehydrogenase, as is the case for *Gluconobacter oxidans* (Buchert 1991). The same a-specific aldolase activity on xylose has also been found in *Klebsiella pneumonia*e (Wang et al. 2016), an opportunistic pathogen found in the commensal human microbiome (Wu et al. 2020). In acidic conditions, *K. pneumoniae* is able to use this non-specific aldolase to form xylonic acid from xylose. In these experimental conditions, *K. pneumoniae* was unable to further degrade xylonic acid. Consequently, xylonic acid accumulated in the culture broth (Wang et al. 2016). It is likely other organisms encoding aspecific aldolases are also able to execute this enzymatic conversion. The excreted xylonic acid can consequently be converted to EG through 2-dehydro-3-deoxy-D-pentonate (2D3DP) which is split to pyruvate and glycoaldehyde which consequently can be converted to glycolic acid and EG (Zhang et al. 2020). Experimental evidence for this metabolic route exists for common inhabitants of the gastro-intestinal tract (Grimont and Grimont 2006; Tenaillon et al. 2010) *Enterobacter cloacae* (Zhang et al. 2020) and *Escherichia coli* (Lu et al. 2021). EG could consequently be converted to ethanol and acetate by PDU BMC-containing microorganisms, such as *P. freudenreichii*, *E. coli,* a variety of *Lactobacillaceae* and *Anaerobutyricum hallii*.

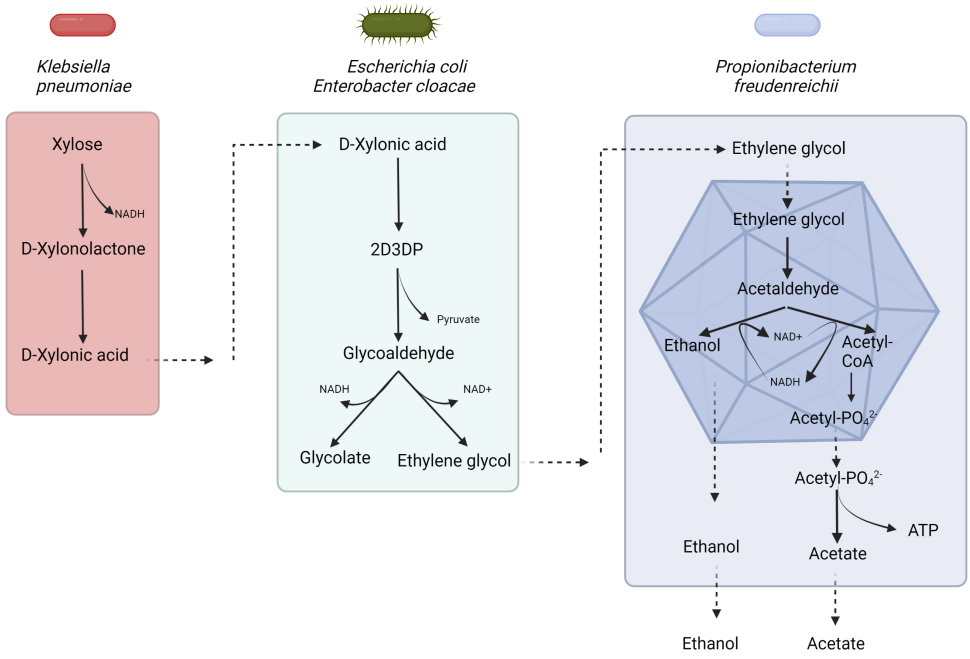

Figure 4.5: Theoretical anaerobic fermentation pathway of xylose to ethanol and acetate within human or animal microbiome involving BMC-mediated degradation of ethylene glycol. Xylose can be metabolized to xylonic acid by bacteria containing a-specific aldolases such as *Klebsiella pneumoniae*, xylonic acid can be converted to pyruvate, ethylene glycol and glycolic acid by *Escherichia coli* and *Enterobacter cloacae* and ethylene glycol can be metabolized to acetate and ethanol by PduCDE containing organisms within a BMC-mediated pathway. 2D3DP = 2-dehydro-3-deoxy-D-pentonate.

Hence, a complete anaerobic pathway (Figure 4.5) allowing degradation of xylan to ethanol and acetate can be constructed within the human microbiome. It is therefore interesting to study whether a gut microbiota consortium would be able to display described activities in order to elucidate the fate of xylan degradation products and its relation to PDU BMC-mediated EG metabolism. It is also interesting to see whether the BMC-mediated EG pathway contributes to bacterial fitness and influences bacterial composition in the human intestine.

Conclusions

P. freudenreichii is able to metabolize EG to acetate and ethanol whilst benefiting energetically. Addition of EG to the growth medium results in significant higher

expression of PDU proteins and DNA repair proteins. As in *A. woodii*, EG is thus metabolized in a PDU BMC-dependent pathway. Evidence of other BMC-containing organisms metabolizing EG with the same diol dehydratase responsible for 1,2-PD metabolism implies the PDU BMC encodes for EG metabolism in multiple organisms. Experimental studies on EG metabolism in PDU BMC-containing organisms and the effect of EG on PDU BMC expression need to be performed to determine whether this phenomenon is widespread. The ability to utilize EG may aid in generation of additional ATP in anaerobic environments with break-down products of xylan, such as the gastro-intestinal tract and thus may play an unexplored role here for PDU BMC encoding microorganisms.

List of abbreviations

1,2-PD: 1,2-propanediol

2D3DP: 2-dehydro-3-deoxy-D-pentonate

A.: Acetobacterium

C.: Citrobacter

E.: Escherichia

EG: Ethylene glycol, 1,2-ethanediol

GRE: Glycyl radical enzyme microcompartment

K.: Klebsiella

L.: Listeria

Li.: Limosilactobacillus

Le.: levilactobacillus

S.: Salmonella

Se.: Secundilactobacillus

PDU: 1,2-propandiol utilization

P.: Propionibacterium

PET: poly(ethylene terephthalate)

BMC: Bacterial microcompartment

T.: Terrisporobacter

Author contributions

AD,RN,XW designed the experimental setup. AD, XW and SB executed the experiments and performed data analysis. AD wrote the initial draft manuscript. All authors read and commented on the draft manuscript. AD rewrote to submitted manuscript. AD, TA, ES revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

All data generated for this study can be found in the additional files and files supplied with this research. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD037369.

Ethics approval and consent to participate

Not applicable.

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Figure 2,3 and 5 were created using biorender.com

Dataset citation

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Supplementary material

Supplementary table 1: Upregulated proteins of *Propionibacterium freudenreichii* grown in Ethylene glycol media compared to *P. freudenreichii* grown in Lactate media. Proteins are upregulated at least 2-fold with a FDR of <0.05.

Protein ID (Uniparc)	Protein Name	Log2 difference	P-value
UPI00005B73B3	1-propanol dehydrogenase PduQ	2.500314	0.0012139
UPI00005B73C1	Ribose-5-phosphate isomerase 3	1.289511	0.0003239
UPI00005B73C4	Transaldolase	1.556124	0.0002050
UPI0001BFF0E9	CoA-dependent propionaldehyde dehydrogenase	7.853431	0.0002685
UPI0001D5C614	Carbon dioxide concentrating mechanism/carboxysome shell protein	6.962153	0.0000002
UPI0001D5C615	Propanediol utilization protein PduB	10.502206	0.0000131
UPI0001D5C616	Glycerol dehydratase large subunit	7.289867	0.0000022
UPI0001D5C682	Transcription regulator	1.188709	0.0028524
UPI0001D5C713	DSBA oxidoreductase	1.353366	0.0007376
UPI0001D5C785	Starvation-inducible DNA-binding protein	1.138147	0.0088891
UPI0001D5C787	Dehydratase small subunit	7.479831	0.0000011
UPI0001D5C78A	Propanediol utilization protein PduK	7.273053	0.0001309
UPI0001D5C79E	Deoxyguanosinetriphosphate triphosphohydrolase (DGTPase)	1.216728	0.0245198
UPI0001D5C8A9	50S ribosomal protein L27	1.134550	0.0051454
UPI0001D5C8AE	Gamma-glutamyl phosphate reductase	1.392323	0.0106010
UPI0001D5CB9A	AMMECR1 domain protein	1.094766	0.0004270
UPI0001D5CBF9	Amino acid permease-associated region	1.061877	0.0099658
UPI0001D5CBFC	Ppx/GppA phosphatase family	1.021743	0.0003320
UPI0001D5CBFD	Uncharacterized protein	1.384352	0.0029133
UPI0001D5CC44	Holliday junction ATP-dependent DNA helicase RuvB	1.142627	0.0012982
UPI0001D5CD00	YhgE/Pip domain protein	1.048417	0.0433454
UPI0001D5CD56	RNA polymerase principal sigma factor HrdD	2.354257	0.0027648
UPI0001D5CD6D	Inositol 2-dehydrogenase IdhA	1.305290	0.0009393

UPI0001D5CD6F	IolC (Myo-inositol catabolism iolC protein)	1.043244	0.0026070
UPI0001D5CD9F	Lactaldehyde dehydrogenase	1.205226	0.0014207
UPI0001D5CDB9	Branched-chain amino acid aminotransferase	1.315311	0.0009116
UPI0001D5CED6	Pyridoxal 5'-phosphate synthase subunit PdxT	4.413929	0.0000103
UPI0001D5CED7	Pyridoxal 5'-phosphate synthase subunit PdxS	4.021846	0.0000025
UPI0001D5CF17	UvrABC system protein B	1.355793	0.0008698
UPI0001D5CF91	Sulfate adenylyltransferase subunit 1	3.762431	0.0001455
UPI0001D5D0FF	Proteasome endopeptidase complex	1.280446	0.0009806
UPI000323070B	Haloacid dehalogenase superfamily enzyme, subfamily IA	1.079201	0.0006389
UPI000323AE06	Diaminopimelate epimerase	1.086419	0.0029175
UPI000324BD76	RNA methyltransferase	1.422096	0.0028494
UPI0003267E56	Propanediol dehydratase medium subunit PduD	9.998640	0.0000028
UPI0004A035A5	ABC-type transport systems, periplasmic component	2.633061	0.0007984
UPI0004A0667C	ATP-dependent DNA helicase	1.049788	0.0009203
UPI0004A0D7A3	Ethanolamine utilization protein EutJ	4.103821	0.0000385
UPI000541B9EB	Uncharacterized protein	3.738367	0.0000031
UPI000541CF70	Formamidopyrimidine-DNA glycosylase (DNA-formamidopyrimidine glycosylase)	2.241622	0.0005575
UPI000541F2C5	DNA repair protein RadA	1.382375	0.0000612
UPI000541F3EC	IolE (Myo-inositol catabolism IolAEprotein) (Inosose dehydratase) (2-keto-myo-inositol dehydratase)	1.356213	0.0085786
UPI0005420BD8	Propanediol utilization diol dehydratase reactivation protein PduG	4.137953	0.0001333
UPI00054232DF	UvrD/REP helicase / ATP-dependent DNA helicase	1.478166	0.0022838
UPI00054244B7	Oxidoreductase	1.602233	0.0000478
UPI000542557F	Corrinoid adenosyltransferase	5.844251	0.0000020
UPI0005428C14	ABC transporter, ATP binding protein	3.216464	0.0000372
UPI0005428DED	Carbon starvation protein	4.182163	0.0020732
UPI0005429954	ABC transporter permease	1.055401	0.0057032
UPI000542A8A2	Propanediol utilization protein PduJ	7.038406	0.0000023
UPI000542B500	Protein-tyrosine phosphatase	1.250518	0.0425564
UPI000542D616	NAD-dependent protein deacetylase	1.122553	0.0037610

UPI0005431885	IolG2 (Myo-inositol catabolism IolG2 protein) (Inositol 2-dehydrogenase)	1.149458	0.0000402
UPI0005435146	IolT3 (Myo-inositol transporter iolT3)	1.310279	0.0002468
UPI00054358A2	ATP binding protein of ABC transporter	1.496289	0.0000275
UPI00054367DC	Probable primosomal protein N'	1.123324	0.0077532
UPI00054370E7	Ethanolamine utilization flavoprotein	5.683172	0.0056373
UPI00054373F7	Sulfite reductase [ferredoxin]	3.203909	0.0002719
UPI0005438BBC	Acetyltransferase family protein	1.614953	0.0043119
UPI000543AC22	Sulfate adenylyltransferase subunit 2	1.590305	0.0047698
UPI000543D7D1	Drug exporters of the RND superfamily	1.571944	0.0000378
UPI000543DF9A	Leucyl/phenylalanyl-tRNA-protein transferase	1.407035	0.0033489
UPI0005440BDD	MEMO1 family protein PFR_JS17-1_19	1.037271	0.0386277
UPI0005441692	Glutamate decarboxylase	1.059917	0.0412921
UPI00054435B7	Two component sensor kinase	1.038407	0.0039921
UPI0005443D9B	Cysteine synthase	2.958105	0.0009408
UPI000544453F	Catalase	1.394799	0.0000458
UPI0005445352	Hypothetical membrane protein	3.100425	0.0000150
UPI0005D83E16	Large conductance mechanosensitive channel<a0>	1.017170	0.0010297
UPI0005DA3579	1-deoxy-D-xylulose-5-phosphate synthase<a0>	1.293938	0.0484307

Supplementary table 2: Proteins upregulated in *Propionibacterium freudenreichii* grown in either 1,2-Propanediol containing media or ethylene glycol containing media compared to lactate containing media. Proteins are upregulated at least 2-fold.

Protein ID (Uniparc)	Protein Name
Pdu Proteins	
UPI0001D5C614	Carbon dioxide concentrating mechanism/carboxysome shell protein PduA
UPI0001D5C615	Propanediol utilization protein PduB
UPI0001D5C616	Glycerol dehydratase large subunit PduC
UPI0003267E56	Propanediol dehydratase medium subunit PduD
UPI0001D5C787	Dehydratase small subunit PduE
UPI0005420BD8	diol dehydratase reactivase PduG
UPI000542A8A2	Propanediol utilization protein PduJ
UPI0001D5C78A	Propanediol utilization protein PduK
UPI0004A0D7A3	Phosphate propanoyltransferase PduL
UPI00054370E7	Propanediol utilization microcompartment protein PduM
UPI0001D5C78E	Propanediol utilization protein PduN
UPI000542557F	cob(I)yrinic acid a,c-diamide adenosyltransferase PduO
UPI0001BFF0E9	CoA-dependent propionaldehyde dehydrogenase pduP
UPI00005B73B3	1-propanol dehydrogenase PduQ
DNA and RNA repair proteins/regulatory proteins	
UPI00054232DF	UvrD/REP helicase / ATP-dependent DNA helicase
UPI0001D5CF17	UvrABC system protein B
UPI000541CF70	Formamidopyrimidine-DNA glycosylase (DNA-formamidopyrimidine glycosylase)
UPI000541F2C5	DNA repair protein RadA
UPI000541B9EB	RtcB family protein
UPI0004A0667C	ATP-dependent DNA helicase
UPI00054367DC	Probable primosomal protein N'
UPI000542D616	NAD-dependent protein deacetylase; Sir2-like transcriptional regulator
UPI0005428DED	Carbon starvation protein
UPI00054435B7	Two component system sensor kinase

Vitamin B6 synthesis	
UPI0001D5CED6	Pyridoxal 5'-phosphate synthase subunit PdxT
UPI0001D5CED7	Pyridoxal 5'-phosphate synthase subunit PdxS
Sulfur metabolism/Protein synthesis/Protein recycling	
UPI0005443D9B	Cysteine synthase
UPI000543AC22	Sulfate adenylyltransferase subunit 2
UPI00054373F7	Sulfite reductase [ferredoxin]
UPI0004A035A5	ABC-type transport systems, periplasmic component; ABC transporter methionine
UPI0001D5C713	DSBA oxidoreductase
UPI0001D5CBF9	Amino acid permease-associated region
UPI0001D5C8AE	Gamma-glutamyl phosphate reductase
UPI0001D5CBA5	50S ribosomal protein L33
UPI000543DF9A	leucyl/phenylalanyl-tRNA–protein transferase
UPI000323AE06	Diaminopimelate epimerase
Putative transporters	
UPI0005428C14	ABC transporter, ATP binding protein
UPI000543D7D1	Membrane protein YdfJ
Various/unknown function	
UPI00054244B7	Oxidoreductase
UPI0005438BBC	Acetyltransferase family protein
UPI0001D5CB9A	AMMECR1 domain protein
UPI0001D5CBFC	Ppx/GppA phosphatase family
UPI0005440BDD	MEMO1 family protein PFR_JS17-1_19
UPI000542DA5A	Methyltransferase type 11
UPI0001D5CD9F	Lactaldehyde dehydrogenase
UPI0001D5CFDE	PF10708 family protein

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5

Microaerobic metabolism of lactate and propionate enhances vitamin B_{12} production in *Propionibacterium freudenreichii*

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Abstract

Background

Propionibacterium freudenreichii is used in biotechnological applications to produce vitamin B_{12}. Although cultured mainly in anaerobic conditions, microaerobic conditions can greatly enhance biomass formation in *P. freudenreichii*. Since B_{12} yields may be coupled to biomass formation, microaerobic conditions show great potential for increasing B_{12} yields in *P. freudenreichii*.

Results

Here we show biomass formation increases 2.7 times for *P. freudenreichii* grown in microaerobic conditions on lactate versus anaerobic conditions (1.87 g/L vs 0.70 g/L). Consumption of lactate in microaerobic conditions resulted first in production of pyruvate, propionate and acetate. When lactate was depleted, pyruvate and propionate were oxidised with a concomitant 6-fold increase in the B_{12} titer compared to anaerobic conditions, showing potential for propionate and pyruvate as carbon sources for B_{12} production. Consequently, a fed-batch reactor with anaerobically precultured lactate-grown cells was fed propionate in microaerobic conditions resulting in biomass increase and production of B_{12}. Vitamin yields increased from 0.3 μg B_{12} per mmol lactate in anaerobic conditions to 2.4 μg B_{12} per mmol lactate and 8.4 μg B_{12} per mmol propionate in microaerobic conditions. Yield per cell dry weight (CDW) increased from 41 μg per g CDW in anaerobic conditions on lactate to 92 μg per g CDW on lactate and 184 μg per g CDW on propionate in microaerobic conditions.

Conclusions

Here we have shown both B_{12} yield per substrate and per CDW were highest on cells oxidising propionate in microaerobic conditions, showing the potential of propionate for biotechnological production of vitamin B_{12} by *P. freudenreichii*.

Background

Vitamin B_{12} (B_{12}), or cobalamin, is an essential vitamin for humans which is exclusively produced by some Bacteria and Archaea. It acts as a co−factor in enzymatic processes, which can be divided into carbon rearrangement reactions, intramolecular methyl transfer reactions and reduction of ribonucleotide triphosphate to 2−deoxyribonucleotide triphosphate. In propionic acid bacteria B_{12} acts as a co-factor in the characteristic Wood-Werkman cycle used to ferment substrates such as lactate. B_{12} is essential in the isomerisation of succinyl-CoA to methylmalonyl-CoA (Marsh et al. 1989), as it acts as a co-factor of methylmalonyl-CoA mutase. B_{12} thus plays an essential role in the main metabolism of propionic acid bacteria under anaerobic fermentation conditions.

B_{12} can be synthesised *de novo* in bacteria through an aerobic and an anaerobic pathway, of which the anaerobic pathway is used by *Propionibacterium freudenreichii* (Moore and Warren 2012; Roessner et al. 2002). Although the B_{12} production pathway in *P. freudenreichii* is anaerobic, yield increments have been reported for *P. freudenreichii* grown under aerobic conditions (Ye et al. 1996). On the other hand, Quesada-Chanto et al. (1998) and Menon and Shemin (1967) found decreased B_{12} production when oxygen was present. The presented studies on B_{12} production have in common that relatively high amounts of oxygen are used, resulting in decreased cytochrome synthesis (Vries et al. 1972; Ye et al. 1996) potentially caused by diminished δ-aminolevulinic acid dehyradatase activity (Menon and Shemin 1967), resulting in lower growth rates and at higher oxygen levels even in diminished growth. Since heme and B_{12} share the same precursors produced by δ-aminolevulinic acid dehyradatase a decreased B_{12} yield could be expected when oxygen diminishes the respective dehyradatase activity (Ye et al. 1999).

Recently Dank et al. (2021) have shown lactate can be completely oxidised using a continuous flow of low amounts of oxygen in a three phase cultivation. Under these conditions, large proportions of lactate are fermented to propionate and acetate, after which when lactate is depleted propionate starts being oxidised

and lastely acetate is being oxidised. The production and subsequent consumption of propionate shown by Dank et al. (2021) can be explained by reversibility of the Wood-Werkman cycle (Emde and Schink 1990) and a functional electron transport chain. Since a functional electron transport chain is required for oxidising propionate with oxygen as terminal electron acceptor, it is conceivable the conditions used by Dank et al. (2021) allow heme, and thus also B_{12} synthesis. As the Wood-Werkman cycle is reversed, methylmalonyl-CoA mutase is still actively required for growth by *P. freudenreichii*. As B_{12} is required as cofactor for methylmalonyl-CoA mutase, reversing the Wood-Werkman cycle may result in a metabolic demand for B_{12} and consequently result in B_{12} production. This raises the question whether B_{12} is still actively produced by *P. freudenreichii* that oxidises propionate. In this study we confirm the 'propionate switch' observed by Dank et al. (2021) in microaerobic conditions on lactate and consequently show microaerobic conditions enhance B_{12} yield on lactate. Furthermore we show propionate can be used as sole carbon source for the production of B_{12} and we show B_{12} yields are drastically improved using propionate as sole carbon source under microaerobic conditions compared to lactate in microaerobic and anaerobic conditions.

Results

Biomass formation and B_{12} yield on lactate drastically increase in microaerobic conditions

P. freudenreichii has been extensively studied as a producer of B_{12} as it favours production of the human active form of B_{12}. *P. freudenreichii* was grown under anaerobic and microaerobic conditions in minimal medium containing lactate (MM-lac) and was sampled at several time points. Biomass formation was found to increase 2.7-fold by microaerobic conditions in MM-lac compared to anaerobic conditions (Figure 5.1A). In anaerobic conditions lactate was metabolised to propionate and acetate in a molar ratio of 1.98:1, close to the theoretical value of 2:1 (data not shown). In microaerobic conditions lactate was metabolised to propionate, acetate and pyruvate (Figure 5.2). Contrary to the results of Dank et al. (2021)

in rich medium, in our chemically defined medium the production of pyruvate was observed and propionate production declined. Biomass formation for anaerobic and microaerobic conditions did not differ significantly (0.38 vs 0.52 g CDW/L, independent student's t-test (p=0.38)) for cells growing on lactate at 48 hours. When lactate was depleted no further biomass formation was observed in anaerobic conditions. In microaerobic conditions depletion of lactate was followed by oxidation of pyruvate and propionate to acetate and CO_2 and a significant (independent student's t-test (p< 0.01)) further increase in biomass (0.70 g/L anaerobic vs 1.87 g/L microaerobic). Total biomass formation after oxidation of propionate and pyruvate thus increased 2.7 times compared to anaerobic conditions, in line with result of Dank et al. (2021), who observed an increase of 2.4.

Oxidation of propionate and pyruvate obviously resulted in an energetic benefit as shown by the increase in biomass formation. The increase in biomass formation was accompanied by a large increase of the B_{12} titer (μg/L), see Figure 5.1B. The B_{12} titer during lactate metabolism in microaerobic conditions (t=48 hours) was found to be similar to that in anaerobic conditions (independent student's t-test (p =0.89)). However, a further increase of B_{12} was observed in microaerobic conditions, whilst in anaerobic conditions the B_{12} yield was minimally increased. The B_{12} titre increased 6-fold in microaerobic conditions compared to anaerobic conditions (p = 0.088, independent student's t-test), which means cells in microaerobic conditions produced on average two times more B_{12}. As shown in Figure 5.1B and Figure 5.2 this may be attributed mainly to the oxidation of propionate and pyruvate. Pyruvate serves as major intermediate metabolite in carbon metabolism and thus is expected to contribute to biomass production and potentially production of B_{12}. Propionate however is considered the metabolic end-product of anaerobic fermentation of lactate in propionic acid bacteria and is not linked directly as major carbon source for the production of biomass and B_{12}. Our results thus raised the question whether propionate would serve as a suitable carbon source for *P. freudenreichii* for biomass formation and production of B_{12} in microaerobic conditions.

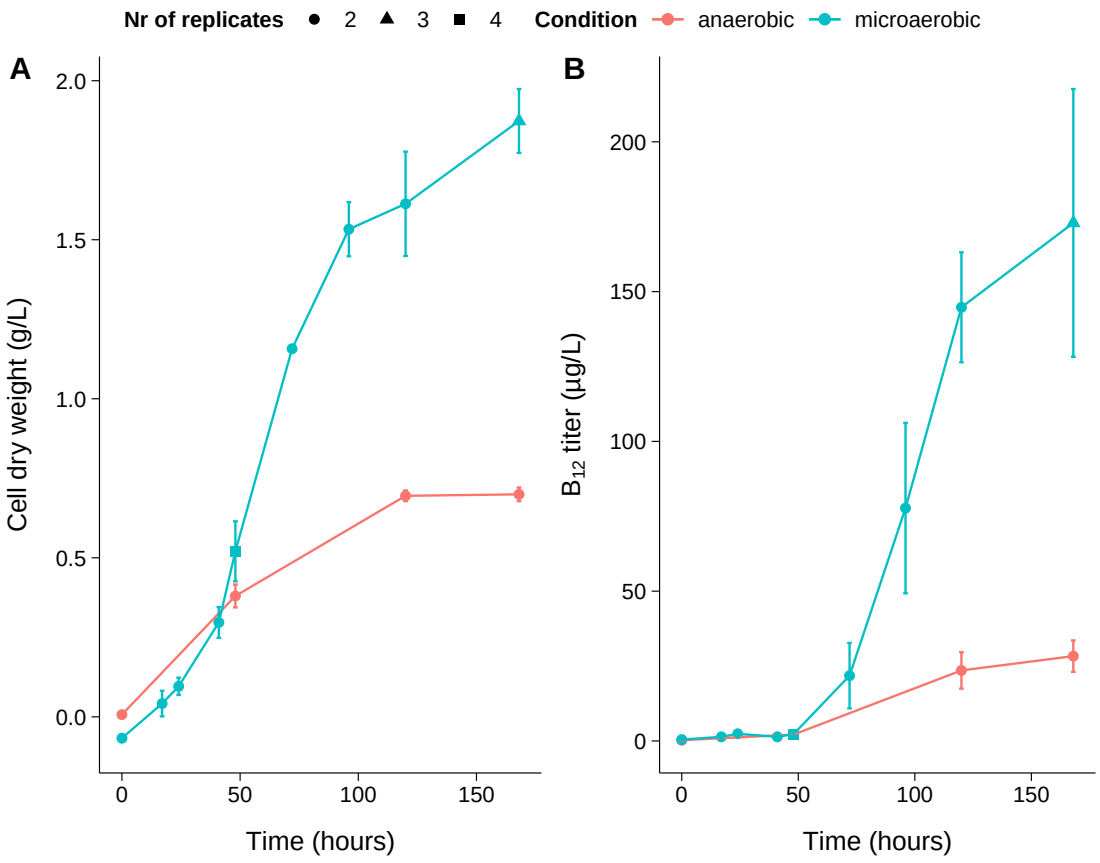

Figure 5.1: Biomass formation (**A**) and B_{12} titer (**B**) in anaerobic and microaerobic conditions for growth of *P. freudenreichii* on lactate. Error bars represent standard error from biological replicates. Number of replicates per timepoint are displayed by circles (n=2), triangles (n=3) or squares (n=4).

Propionate oxidation supports biomass formation and B_{12} production

To study whether *P. freudenreichii* can grow on propionate as carbon source a bioreactor setup using minimal medium containing 100 mM propionate (MM-prop) was attempted. Surprisingly, no growth was observed under these conditions which may be attributed to a combination of inhibition by propionate (Martinez-Campos and Torre (2002)), toxicity by oxygen (Vries et al. (1972)) and low inoculum (see discussion). To minimise the product inhibition of propionate a fed-batch reactor was set up. Cells were pre-cultured in anaerobic conditions on lactate and transferred to a bioreactor with MM-propionate in microaerobic conditions. The inoculum size to the bioreactor was increased from 2% (v/v) to 10% (v/v). Propionate was fed to

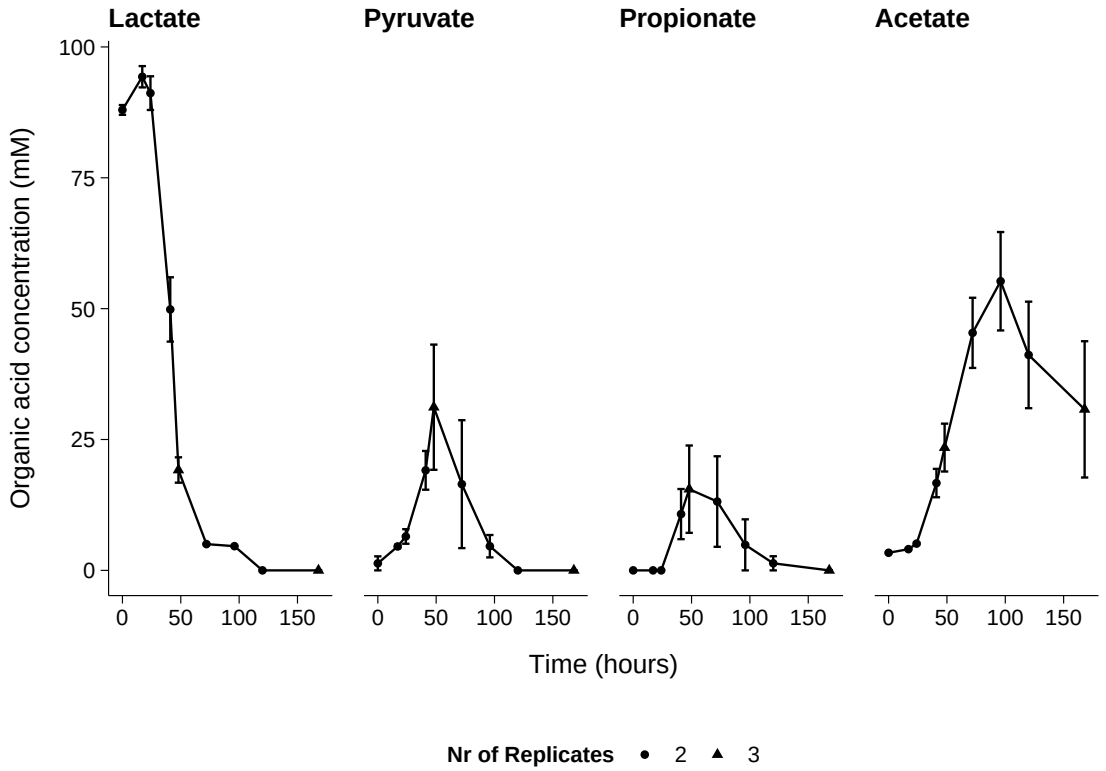

Figure 5.2: Substrate consumption and primary metabolite production in microaerobic conditions for *P. freudenreichii* growing on lactate. Error bars represent standard error from biological replicates. Number of replicates per timepoint are displayed by circles (n=2) and triangles (n=3).

these cells to a final concentration of 10 mM at specific time points (t=0 h, t=48 h, t=120 h and t=168 h) whilst keeping the flux of oxygen constant. Injection of cells lead to consumption of oxygen (Figure 5.4) and complete consumption of propionate with a concomitant increase of biomass and B_{12} as shown in Figure 5.3. The oxidation of propionate resulted first in the formation of acetate (data not shown). Oxygen was readily consumed after the primary injection and remained at the lower detection limit until depletion of both propionate and the formed acetate, after which oxygen levels steadily rose again. Injection of fresh propionate resulted in instantaneous consumption of oxygen, which confirmed respiratory pathways were used for metabolism of propionate and acetate with oxygen as terminal electron acceptor (Figure 5.4). These results also indicate no loss of electron transport chain functionality at oxygen fluxes used in our studies.

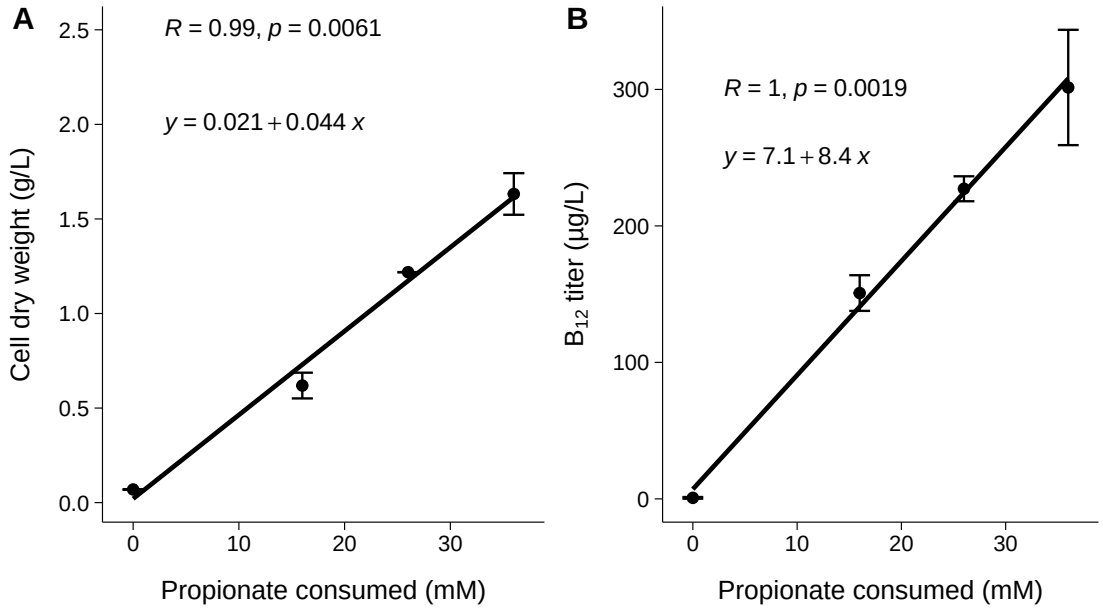

Figure 5.3: Biomass formation (A) and B_{12} titer (B) in microaerobic conditions for *P. freudenreichii* growing on propionate in a fed-batch reactor. Error bars represent standard error from biological duplicates.

Propionate is the substrate with the highest B_{12} yield per substrate and per biomass

The propionate fed-batch cultivation clearly shows the potential of propionate as a carbon source for B_{12} production. To compare the B_{12} yield on different substrates correctly the B_{12} yield per substrate was calculated at 168 hours (Figure 5.5). A 7.5-fold increased yield per substrate was found for microaerobic lactate-grown cells versus anaerobic-grown cells. An increased yield of 26.3 times was found for microaerobic propionate-grown cells versus anaerobic lactate-grown cells. The yield per CDW increased two-fold for propionate-grown microaerobic cells versus lactate-grown microaerobic cells and 4.5 times for lactate-grown anaerobic cells. Both the productivity per substrate ($p<0.01$ for propionate as substrate, multiple linear regression) and productivity per cell biomass ($p<0.05$ for propionate as substrate, multiple linear regression) thus increases drastically when metabolising propionate in microaerobic conditions compared to lactate in anaerobic and microaerobic conditions.

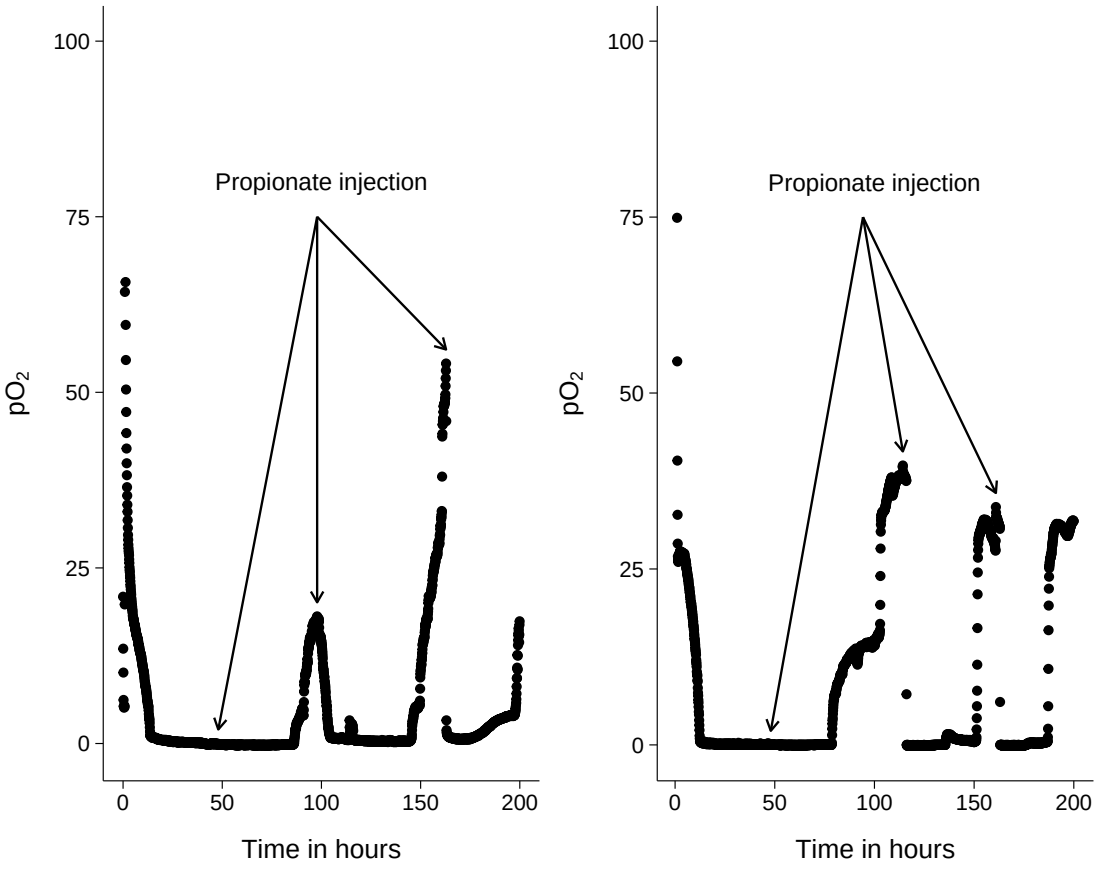

Figure 5.4: Dissolved oxygen measurement in bioreactors throughout cultivation on propionate. Dissolved oxygen as expressed as percentage of content measured at 100 percent air at 0.1 $L * min^{-1}$ at 30 degrees using 300 RPM and 0 percent air. Arrows indicate at which time new propionate was injected to an end concentration of 10 mM. Samples for biomass, HPLC and B_{12} quantification were taken directly prior to each new propionate injection.

Discussion

P. freudenreichii has been extensively studied as a producer of B_{12} as it favours production of the human active form of B_{12} (Deptula et al. 2015) and has the generally recognised as safe status (Falentin et al. 2010). Many different strategies for increasing B_{12} yield by *P. freudenreichii* have been attempted, such as genetic engineering (Piao et al. 2004), genome shuffling (Zhang et al. 2010), media optimalisations (Komider et al. 2012) and changing environmental conditions such as presence or absence of oxygen (Ye et al. 1996) or activation of riboswitches using blue light (Yu et al. 2015). In anaerobic processes the production of propionic

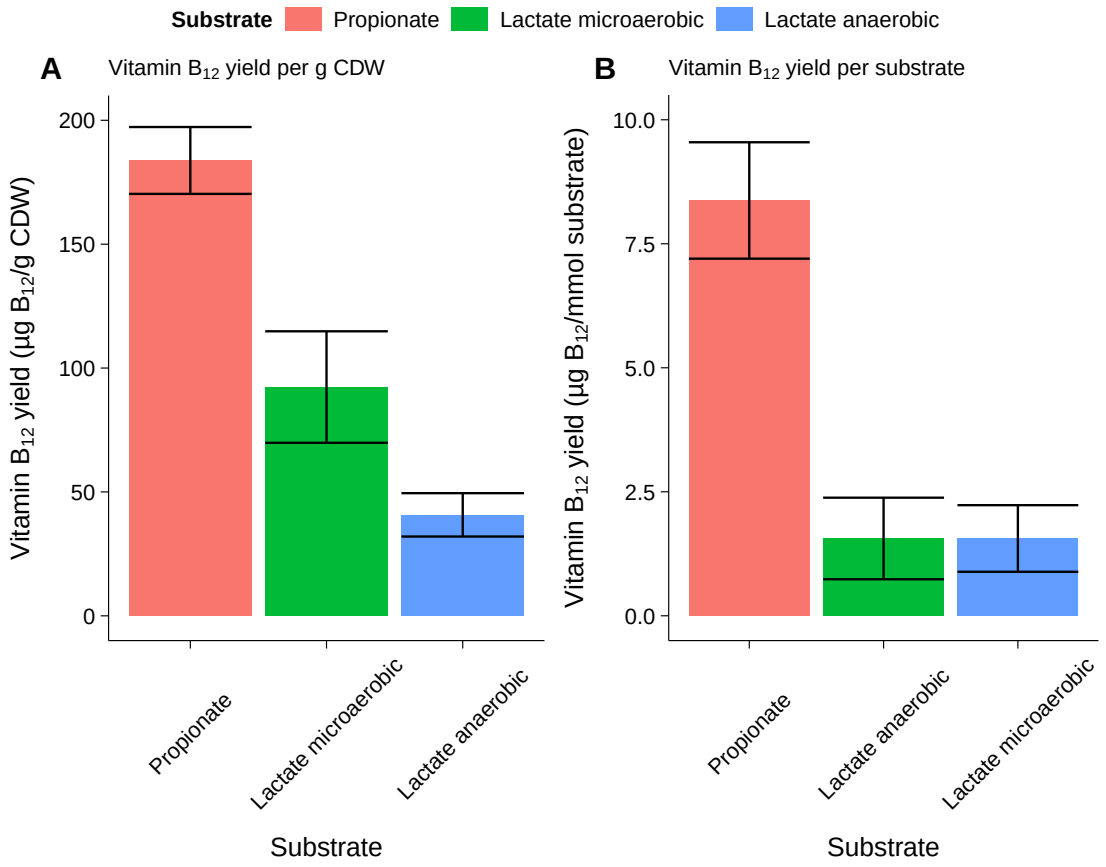

Figure 5.5: B_{12} yield per g cell dry weight and per mmol substrate for *P. freudenreichii* in anaerobic and microaerobic conditions. Error bars represent standard error from biological duplicates.

acid (and conceivabily acetic acid (Pinhal et al. 2019)) by *P. freudenreichii* causes product inhibition, resulting in decreased cell growth and B_{12} synthesis (Wang et al. 2015; Ye et al. 1996). However, a decreased B_{12} yield per gram cells has also been reported for processes removing propionic acid efficiently (Wang et al. 2012). Indeed, Wang et al. (2015) have found maintaining propionic acid concentrations at specific levels can increase B_{12} production. The role and effect of propionic acid on final B_{12} yield thus remains complex, but point towards higher production of B_{12} with minimal presence of propionate in the environment. What most studies have in common is a goal to remove propionic acid (and acetic acid) or decrease its negative effect on cell growth in anaerobic conditions. Usually, this is done by mechanical means such as removing effluent whilst returning or immobilizing cells (Miyano et al. 2000; Yongsmith et al. 1982). Here we attempt to solve this problem

in a bioenergetically favourable way; removal of propionic acid (and acetic acid) by oxidation, resulting in ATP generation and lower inhibition potential and possible activation of pathways requiring B_{12}, such as the Wood-Werkman cycle.

Results obtained in the current study support the findings of Dank et al. (2021) in chemically defined lactate medium in microaerobic conditions and show that during these conditions biomass production and B_{12} production drastically increases. However, contrary to Dank et al. (2021) we also observed accumulation of pyruvate. Similar observations were found for *Acidipropionibacterium acidipropionici* by Gent-Ruijters et al. (1976), who attribute the accumulation of pyruvate to a lack of oxidative decarboxylation of pyruvate. Indeed, oxygen inhibits pyruvate-ferredoxin oxidoreductase (PFOR) (Lu and Imlay 2021; Pan and Imlay 2001), which has been proposed to be a key enzyme during the utilization of lactate by propionic acid bacteria (McCubbin et al. 2020). Alternatively, in microaerobic conditions pyruvate may be directly oxidised using oxygen as acceptor by pyruvate oxidase (PO), producing CO_2, H_2O_2 and acetyl-phosphate (McCubbin et al. 2020). Consequently if the anaerobic route for pyruvate dissimilation is disabled due to inactive PFOR, accumulation of pyruvate can be expected when oxygen contents are limited and pyruvate dissimilatory pathways requiring oxygen directly (PO) or indirectly (through oxygen-dependent NADH:dehydrogenase activity (pyruvate dehydrogenase)), are limited in flux and compete with other processes requiring regeneration of NADH to NAD^+. This hypothesis is supported by the observations of Ye et al. (1999), who reported accumulation of pyruvate after injection of propionate in microaerobic conditions, implying the rate-limiting step during propionate oxidation to acetate occurs at the pyruvate node. Therefore, the most likely explanation is a stochiometric limitation of oxygen, limiting the amount of oxygen available for NADH dehydrogenase-coupled electron transport activity in combination with potential competition for oxygen by pyruvate oxidase and (partial) inactivation of other key metabolic enzymes such as PFOR, resulting in small NAD^+ pools and pyruvate accumulation and production of propionate. The described stochiometric limitations in oxygen levels are in line with the reported

sensitivity of *P. freudenreichii* to oxygen, while efficient substrate metabolism is supported in microaerobic conditions.

In our study propionate oxidation in microaerobic conditions resulted in a boost of B_{12} production, while in previous studies Ye et al. (1996) observed ceased B_{12} production conceivably due to loss of δ-aminolevulinic acid dehydratase activity (Menon and Shemin 1967) and consequently loss of cytochrome synthesis (Vries et al. 1972) in the high oxygenation conditions used in their experiments. Our results suggest it is key to keep oxygen fluxes low in order to maintain the ability to oxidise substrates using oxygen as a terminal electron acceptor. These results are supported by results of Tangyu et al. (2022), who report highest B_{12} production at specific oxygen regimes in their food product. Since oxygen is required as terminal electron acceptor for oxidation of propionate, constraining oxygen to low levels results in oxygen being the growth rate determining factor. Since oxygen is supplied at a constant rate, the observed growth of *P. freudenreichii* on propionate in microaerobic conditions is linear (Dank et al. 2021). Hence, to increase biomass formation and B_{12} production in time, higher oxygen fluxes should be applied, which requires increasing aerotolerance and/or respiration rates in *P. freudenreichii.* It is therefore interesting to attempt to obtain mutants with increased respiration rates by genetic engineering or adaptive evolution approaches. We hypothesised that the utilisation of propionate as sole carbon source for *P. freudenreichii* in microaerobic conditions, will result in forcing flux through the reversed Wood-Werkman cycle. This in turn will result in a demand for B_{12} in growing cells as co-factor in the methylmalonyl-CoA transferase reaction and consequently activation of B_{12} production. However, in our first setup using 100 mM propionate in combination with the same microaerobic conditions applied on lactate no growth was observed. Both propionate (Martinez-Campos and Torre 2002) and oxygen (Vries et al. 1972) are known to be toxic for *P. freudenreichii.* Since the same microaerobic conditions were used as in the experiments on lactate as a carbon source, oxygen itself is not deleterious enough to inhibit growth at the used oxygen regime. Furthermore, Dank et al. (2021) have shown that propionate is oxidised at higher concentrations (~70 mM) when larger

amounts of biomass are present and pO_2 inside the system is 0. Initial cell numbers are also reported to influence the potential of *P. freudenreichii* to either grow or not grow in milk (Piveteau et al. 2000). Environmental stresses limit microbial growth in either synergistic or even multiplicative manner (Biesta-Peters et al. 2010; Leistner and Gorris 1995). Hence, it is conceivable the imposed stress of both propionate and oxygen in our initial setup was too big of a 'hurdle' for the low inoculum used in our study. It is therefore key to minimise the imposed stresses on *P. freudenreichii* by using a combination of low oxygen fluxes, low propionic acid concentrations and high initial biomass numbers.

Using a fed-batch system and thus low concentrations of propionate (10 mM), thereby preventing excessive product inhibition, we provide evidence that oxidation of propionate leads to production of acetate, which was further oxidised to CO_2 during prolonged incubation. The oxidation of propionate to acetate and consequently to CO_2 leads to a significant increase of B_{12} production per cell biomass, i.e., an increased yield per g CDW of 184 μg/g CDW on propionate versus 92 μg/g CDW on lactate microaerobically and 41 μg/g CDW on lactate anaerobically. The presence of propionate as sole carbon source thus increased the yield considerably. To conclude, we have shown that propionate can serve as excellent carbon source for *P. freudenreichii* in microaerobic conditions. This opens up great potential for the application of microaerobic conditions in combination with controlled propionate feeding for efficient production of B_{12}. Applications of (genetically) engineered B_{12}-overproducing strains (Piao et al. 2004; Zhang et al. 2010) in combination with other optimisation strategies such as media optimisations (Komider et al. 2012) by addition of precursors or altering other environmental conditions such as light (Yu et al. 2015) as previously suggested are recommended to investigate further yield increments using propionate as substrate in microaerobic conditions. Further studies about the regulatory role of propionate in activation of B_{12} biosynthesis pathways in *P. freudenreichii* can provide clues for further optimisation.

Conclusions

Here we show minimal fluxes of oxygen can greatly enhance biomass and B_{12} production in *P. freudenreichii* with lactate or propionate as a substrate. Stochiometric constraints of oxygen cause triauxic growth on lactate as observed earlier by Dank et al. (2021). The formation and consequent oxidation of propionate appeared to be linked to increasing B_{12} titer and yield. Fed-batch experiments showed that propionate can serve as excellent carbon source for biomass production and B_{12} production. Further studies on the potential regulatory role of propionate in activation of B_{12} synthesis in *P. freudenreichii* need to be performed. Since the oxidation of propionate is limited by the stochiometric constraint of oxygen, optimising oxygen fluxes, increasing aerotolerance and/or respiration rates in *P. freudenreichii* may aid in improving oxidation rates and thus biomass and B_{12} production.

Methods

Strain and preculture conditions

P. freudenreichii DSM 20271 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and routinely grown on yeast extract lactate (YEL) consisting per liter of: 10 g tryptone, 5 g yeast extract, 5 g KH_2PO_4 and 16 g 80% L-Lactate syrup (Sigma Aldrich) and 15 g bacteriological agar for plates. Cell cultures were grown for 3 days in liquid media and maintained in 30% (v/v) glycerol stocks at -80 °C. Cells were precultured for each experiment by streaking *P. freudenreichii* on YEL agar and incubating at 30 °C in anaerobic conditions for 7 days. Single colonies were inoculated in minimal medium with composition described below.

Minimal media

Minimal media (MM) used in this study consisted per liter of : 100 mM carbon L-lactate (MM-lac), 10 mL metal stock(100x), 10 mL nucleic acid stock(100x), 10

mL vitamin stock(100x) and 400 mL amino acids stock(2.5x) with the following compositions for each stock described below. Metal stock per kg: $MgCl_2.6H_2O$ 20 g, $CaCl_2.2H_2O$ 5 g, $ZnSO_4.7H_2O$ 0.5 g, $CoCl_2.6h_2O$ 0.25 g, $MnCl_2.4H_2O$ 1.6 g, $CuSO_4.5H_2O$ 0.25 g, $(NH_4)6Mo_7O_{24}.4H_2O$ 0.25 g, $FeCl_3.6H_2O$ 0.3 g, $FeSO_4.7H_2O$ 0.3 g ($FeSO_4.7H_2O$ was first dissolved in 10 ml 17% HCl, before it was mixed with the other compounds). Nucleic acid stock per kg: 1 g of each dissolved in 0.1 M NaOH; adenanine, uracil, xanthine, guanine. Vitamin stock per kg: Ca-d-pantothenate 0.1 g, d-biotin 0.25 g, thiamin-HCl 0.1 g, na-p-aminobenzoate 1 g. Amino acids stock: 1 mM of L-Alanine, L-Arginine Hydrochloride, L-Asparagine monohydrate, L-Aspartic Acid, L-Cysteine hydrochloride, L-Cystine, L-Glutamic Acid, L-Glutamine, Glycine, L-Histidine hydrochloride, L-Isoleucine, L-Leucine, L-Lysine hydrochloride, L-Methionine, L-Proline, L-Serine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine. For fed-batch experiments L-lactate was replaced by 10 mM propionate (MM-prop).

Bioreactor cultivations on lactate

Bioreactor cultivations were performed according to the methods described by Dank et al. (2021). A single colony of *P. freudenreichii* was inoculated from YEL agar plates in 10 mL MM-lac and incubated at 30 °C anaerobically for 5 days, after which 2% (v/v) was inoculated into bioreactors with a working volume of 500 mL (Multifors, Infors HT, Switzerland). The stirring speed was set at 300 rpm, the temperature was kept constant at 30°C and the pH was controlled at 7.0 by automatic addition of 5 M NaOH and 1 M HCl. A gas mix containing 85% N_2 gas and 15% air was used for microaerobic conditions. Gas was supplied through a sparger at the bottom of the fermenter using a mass flow controller premixing gas at set values at a rate of 0.1 L/min. Dissolved oxygen was measured using a probe which was calibrated at 100% by flushing the system with pure air at 0.1 L/min for 2 hours and at 0% by flushing the system with N_2 for 2 hours. Samples were taken at various time points aseptically through a sampling port. *P. freudenreichii* was grown in anaerobic conditions in 50 mL greiner tubes in

MM-lac as described above and sampled at the several timepoints as reference condition. All samples were stored at -20 °C.

Fed-batch cultivations on propionate

P. freudenreichii was precultured on MM-lac in anaerobic conditions as described before. *P. freudenreichii* was inoculated into bioreactors containing 10 mM propionate minimal medium (MM-prop). Bioreactor settings were equal to settings used for cultivation on lactate described above. 10 mL samples were taken at 0 h, 48 h, 120 h and 168 h. After each sample point a new injection with 10 mL of 500 mM propionate stock was made to establish an end concentration of 10 mM propionate in the reactor after the injection. All samples were stored at -20 °C

Biomass quantification

Biomass was quantified by measuring the cell dry weight (CDW) concentration as described by Mastrigt, Abee, et al. (2018). Membrane filters with a pore size of 0.2 μm (Pall Corporation, Ann Arbor, MI, USA) were pre-dried in an oven at 80 °C and then weighed. Samples were passed through the pre-weighted membrane filters using a vacuum filtration unit. Residual cell material in the funnel was washed to the filter using approximately 30 mL of demi water. The filters containing the biomass were dried at 80 °C again, after which filters were weighed again. CDW was calculated using the following formula:

$$CDW\ (\frac{g}{kg}) = \frac{Weight\ filter\ + biomass\ (g) - weight\ filter(g)}{Amount\ of\ culture\ (g)} * 1000$$

Analysis of organic acids

Lactate, acetate, propionate and pyruvate were quantified by High Performance Liquid Chromatography (HPLC) as described by Mastrigt, Mager, et al. (2018). Briefly, 500 μL of sample was deproteinized by addition of 250 μL Carrez A (0.1 M potassium ferrocyanide trihydrate), mixing, addition of 250 μL Carrez B (0.2

M zinc sulfate heptahydrate), mixing and centrifugation for 2 minutes at 17000 x g. 200 μL supernatant was injected on a UltiMate 3000 HPLC (Dionex Germany) equipped with an Aminex HPX-87H column (300 x 7.8 mm) with guard column (Biorad). 5 mM H_2SO_4 was used as mobile phase with a flow rate of 0.6 mL/min at a column temperature of 40 °C. Compounds were detected using a refractive index detector (RefractoMax 520).

B_{12} quantification

B_{12} was detected using a Vitafist B_{12} biological assays (R-Biopharm). Samples were prepared for analysis by diluting them 10x with demi water. Samples were beat-beaded in FastPrep-24 instrument (MP Biomedicals) 3 times using 1-minute intervals followed by centrifugation at 17000 x g for 2 minutes. Supernatants were collected and diluted 4x with demi water, after which they were heated for 30 minutes at 95 °C in a water bath. Samples were chilled on ice and diluted further to fall within the microbiological assay detection range. B_{12} assays were performed as described by the manufacturers protocol in technical replicates. Absorbance was measured in microtiter plates using Microwell Plate Reader SpectraMax M2 at 610 nm with SoftMax Pro software.

Statistical analysis

Statistical analysis was performed using R in combination with Rstudio. Data normality was tested using Shapiro-Wilk test. Equal or unequal variance was tested using F-tests. Both normality and equal variances were assumed when $p>0.05$. Independent-students t-tests were used based on equal variances using R t.test function. The effects of microaerobic conditions and propionate as substrate were estimated using multiple linear regression in R using the lm function. Both yield per substrate and per cell were fitted using substrate and condition as dependent variables.

List of abbreviations

P. = *Propionibacterium*

CDW = cell dry weight

Vitamin B_{12} = B_{12}

MM = minimal medium

DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen

YEL = yeast extract lactate

Author contributions

AD, GB, TA and ES designed experiments. AD and GB carried out experiments and analysed the data. AD wrote the draft manuscript. All authors read and commented on the draft manuscript. AD wrote the final version of the manuscript and all authors read and approved submission of the draft manuscript. AD, ES, TA revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article (and its additional file(s)) and can be found online at: https://doi.org/10.1186/s12934-022-01945-8.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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6

Cross-over fermentations and their potential in product development

A. Dank (2022)

This chapter consists of two case studies.

The dairy miso case study is published as:
A. Dank, O. van Mastrigt, Z. Yang, V. M. Dinesh, S. K. Lillevang, C. Weij, and E. J. Smid (2021). The cross-over fermentation concept and its application in a novel food product: the dairy miso case study. *LWT* 142:111041

Manuscript in preparation for lupin quark case study:
T. Canoy, J. Zwinkels, J. Wolkers-Rooijackers, A. Dank, J. van Wiggen, T. Abee, E.J. Smid (2022)

Abstract

Cross-over fermentations are processes in which a microorganism from one traditional fermentation process is introduced onto a new substrate and/or to a new microbial partner. When designed correctly, these novel combinations can result in fermented food products with enhanced product characteristics, such as enhanced nutritional value or aroma/flavor development. Here, we discuss two examples of cross-over fermentations and show the benefits of applying this new concept in novel product development. The first example covers the production of lupin quark using two lactic acid bacteria and *in situ* biofortification of this lupin quark by fermentation of *Propionibacterium freudenreichii*. *P. freudenreichii* was able to ferment lupin milk in mono and in co-cultures, whilst producing ~3 μg of B_{12} per 100 gram of product. The second example covers the development of a novel food product called dairy miso, produced by fermenting quark with *Aspergillus oryzae*, resulting in a food product rich in aroma. These examples show the power of cross-over fermentations in novel product development or enhancement of product characteristics. The enormous diversity of microorganisms used in traditional fermentation processes and the vast number of alternative substrates offer numerous opportunities for the development of novel fermented products.

Introduction

Many classical food products in the world, like bread, cheese, wine, yoghurt, olives, vinegar, beer, tea, tempeh, soy sauce and many more are fermented (McGee 2007). Fermentation is ubiquitously present in all cultures, which all use their own raw materials and microbiological workhorses to convert it to the often so-typical products belonging to that culture. The periodic table of fermented foods by Gaenzle (2015) nicely shows this large variety of food fermentations carried out across the globe. Although food fermentations have been around for centuries, the role of microbial life in fermentation was not known until the discovery of fermentation by Louis Pasteur (Bordenave 2003). Research regarding fermentations led to the isolation of dominant species of bacteria and yeasts present in these fermentations and ultimately to the use of single strain or mixed-culture defined starter cultures to start the fermentation process. The use of defined starter cultures led to better product quality control and higher product consistency (Ross et al. 2002). However, a clear drawback of using single strain defined starters is the loss of microbial diversity in fermented foods and restriction of production of (beneficial) end-products limited to the metabolic repertoire of the used starter. Potentially, the industrialisation of artisanal fermentation processes thus decreases food functionality. This is exemplified by the fact that vitamin B_{12} presence in tempeh can be attributed to the presence of a (harmful) contaminant microbe and is thus not associated with the starter mold (Areekul et al. 1990). The use of good-manufacturing practices and starter cultures thus improves food safety, but potentially decreases food functionality. This hurdle could be overcome by replacing the contaminant bacteria with a safe alternative that also produces the beneficial compound, such as microorganisms with historical evidence of safe usage, the so-called GRAS (generally recognised as safe) status. Valuable sources of beneficial microorganisms might be other, distinctly different, fermented food products. The introduction of a microorganism from a traditional fermentation process to novel substrates and/or to a new fermentation partner is what we coined “cross-over fermentations” (Dank,

Mastrigt, Yang, et al. 2021). Considering the large metabolic diversity found in artisanal fermented foods, there is a large potential for novel fermented food product development and fermented food product enhancements that needs to be explored.

In this chapter we aim to show the potential of cross-over fermentations by showing two examples of such fermentations. In the first example, cross-over fermentation is used to nutritionally enhance a previously in-house designed plant-based fermented food (Canoy 2021). *Propionibacterium freudenreichii*, normally used in Swiss-type cheese fermentations, is applied to a plant-based product to enhance the B_{12} content. In the second example cross-over fermentation is used to develop a novel food product with interesting organoleptic properties. *Aspergillus oryzae* is used on a dairy substrate to create a novel fermented dairy product with rich aromatic properties. A discussion on the benefits and drawbacks of the utilization of single-strain or multi-strain defined starter cultures is given to put the potential of cross-over fermentations into perspective.

Enhancing nutritional quality of fermented foods by cross-over fermentation: The lupin quark case study

Due to increasing world population numbers, consequent increased use of agricultural land and natural resources and increased carbon emissions the need for more sustainable dietary patterns is emerging. A large contributor to global greenhouse gas emissions is meat production (Steinfeld et al. 2006). Reducing global meat production thus could contribute significantly to reducing global greenhouse gas emissions. However, plant-based foods and dietary patterns are associated with a reduced uptake of several important micro nutrients, such as vitamin B_{12} (Neufingerl and Eilander 2021). One way to combat this, is to fortify plant-based foods by fermentation with producers of beneficial compounds. Vitamin B_{12} is produced *de novo* by prokaryotes, such as *Propionibacterium freudenreichii* (Deptula et al. 2015). Below we show an example of the development of a B_{12} fortified plant-based quark, made with lupin beans. A combination of *Lactococcus cremoris* and

Lactobacillus plantarum was used to produce the initial lupin quark product, as designed previously in our laboratory (Canoy 2021). Here we assayed whether *P. freudenreichii* could be used to fortify this product with vitamin B_{12}. Important parameters such as acidification, metabolite production, population dynamics and vitamin B_{12} content were monitored.

Mono and co-cultures of *P. freudenreichii* are able to ferment lupin milk into quark

Lupin milk was inoculated with mono or co-cultures of *P. freudenreichii, L. cremoris* and *Lb. plantarum*, respectively and pH was monitored for a period of 4 days (see Figure 6.1). All bacteria were able to acidify lupin milk. Amongst the mono-cultures, *Lb. plantarum* showed fastest acidification (pH 4.7± 0.03 after 24h) which remained stable until the end of the fermentation. Both *P. freudenreichii* and *L. cremoris* showed slower acidification after 24h (pH 5.9± 0.06 and 5.8± 0.04 respectively) and had higher pH at day 4 (pH 5.2± 0.3 and pH 5.1± 0.1 respectively). Co-culture of *L. cremoris* and *Lb. plantarum* showed similar trends as mono-culture of *Lb. plantarum* reaching similar final pH (4.6± 0.05). Interestingly, all co-cultures containing *P. freudenreichii* reached a final pH between 5.1 and 5.4, which was significantly higher compared to mono-cultures of lupin milk fermented with *Lb. plantarum* and co-cultures containing *Lb. plantarum* (Tukey HSD, $p<0.05$). This is likely due to consumption of lactate and release of less acidic organic acids acetate and propionate into the lupin milk. Lactate has a pKa value of 3.86, propionate and acetate have a pKa value of 4.75 and 4.76 respectively, meaning lactate is 10x more acidic than propionate and acetate and consumption of lactate and full conversion of propionate to acetate would increase pH by 1 point respectively (disregarding potential differences in release of other components affecting pH such as NH_3). The slower acidification rate and higher final pH obtained with *P. freudenreichii* may have implications for product stability and safety.

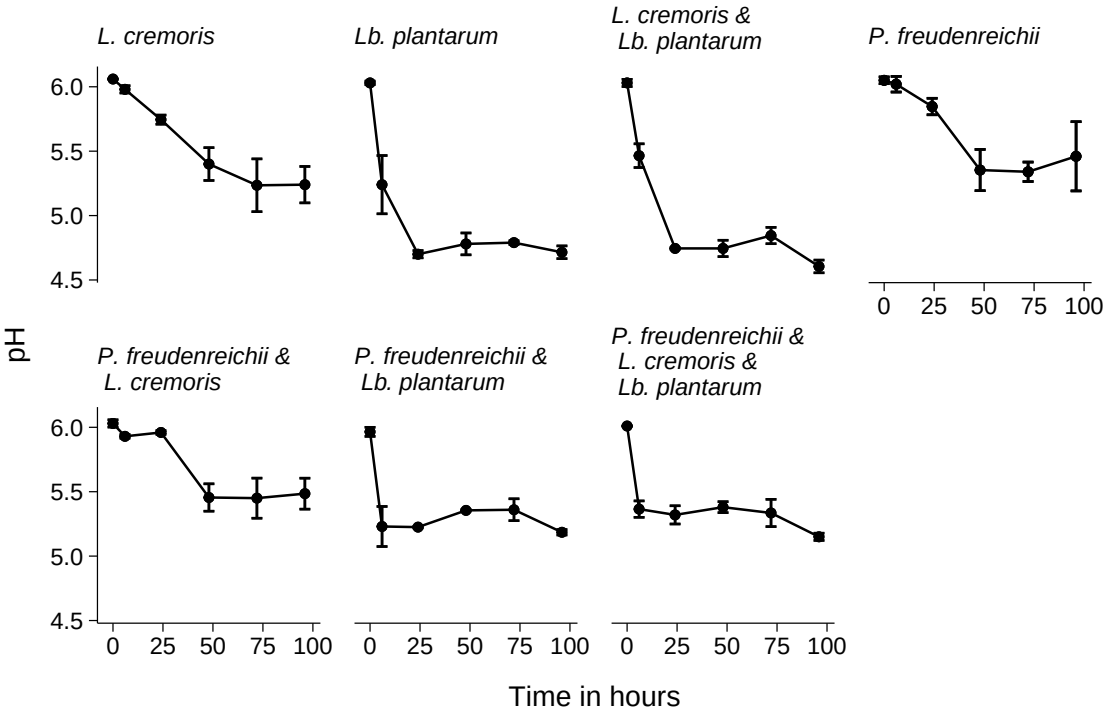

Figure 6.1: Acidification profiles of lupin quark.

Carbohydrate consumption and organic acid profiles of lupin quark

We measured the initial content of several carbohydrates in lupin milk; galactose (1.7 mM), glucose (0.7 mM), fructose (0.3 mM), sucrose (2.1 mM) and raffinose (0.4 mM) were quantified. These carbohydrates may act as primary carbon sources for *L. cremoris, Lb. plantarum* and *P. freudenreichii*. *L. lactis* is able to utilize fructose, galactose, glucose and sucrose, but is not able to utilize raffinose (Canoy 2021). *Lb. plantarum* is able to utilize fructose, galactose, glucose, raffinose and sucrose (Canoy 2021). *P. freudenreichii* is able to utilize fructose, galactose and glucose (Borghei et al. 2021), but is not able to utilize sucrose and raffinose (Tarnaud et al. 2020). Our results support these claims, as we found all three bacteria to consume fructose, galactose and glucose, whilst raffinose utilization was not apparent for any of the bacteria (data not shown) and sucrose only for the LAB. Hence, competition between these carbon sources in co-cultures is conceivable (discussed below).

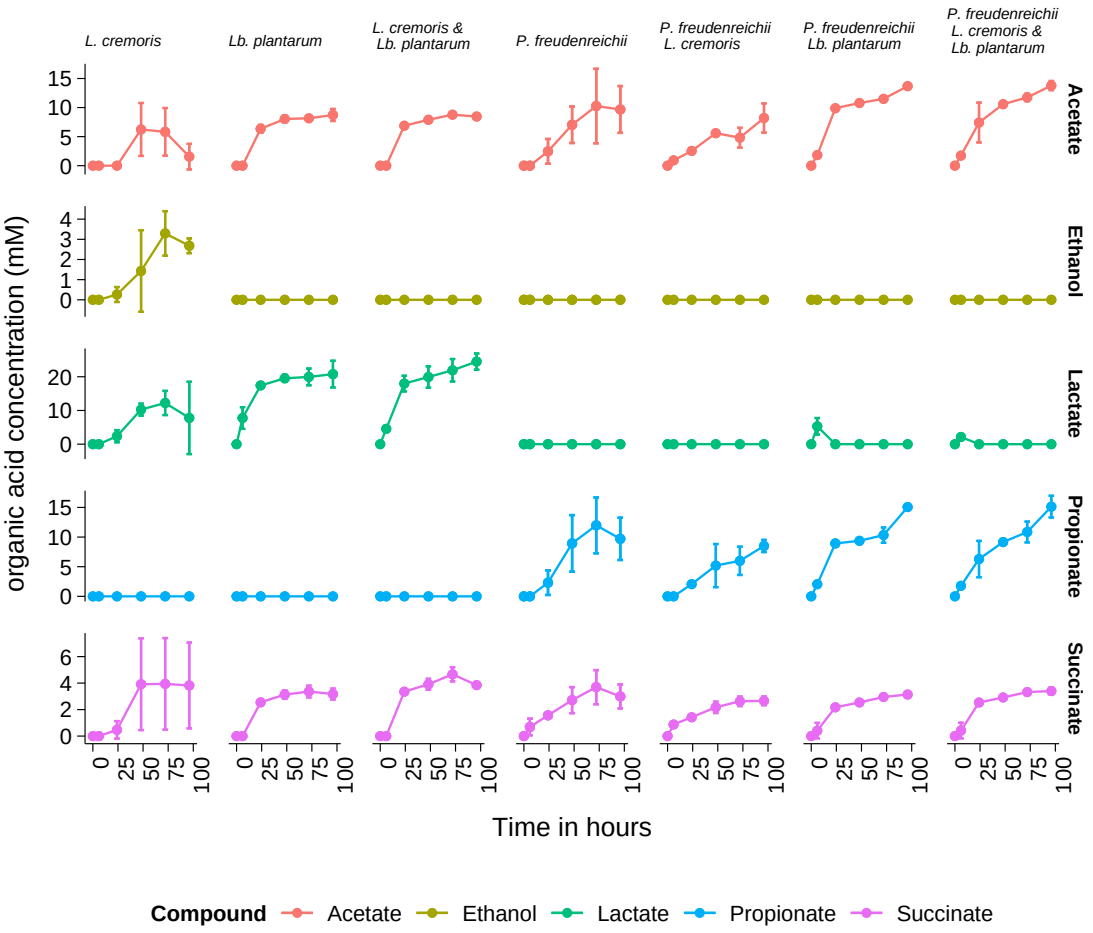

Figure 6.2: Organic acids and ethanol formation in lupin quark.

Fermentation of fermentable carbon sources in lupin milk resulted in the production of organic acids and ethanol (see Figure 6.2). All species produced acetate and succinate in mono-culture. Both *L. cremoris* and *Lb. plantarum* produced lactate in mono-culture, whereas ethanol was only produced by *L. cremoris* and propionate by *P. freudenreichii* in mono-culture. Interestingly, when *L. cremoris* was growing in co-cultures ethanol could not be detected anymore. Furthermore, when assuming cumulative production of lactate and acetate by *L. cremoris* and *Lb. plantarum* in co-culture, higher end-values of these compounds are expected based on the production of these compounds in mono-culture. This indicates there is competition between *Lb. plantarum* and *L. cremoris* for the same carbon sources. Interestingly, in co-cultures containing *P. freudenreichii* lactate was hardly detected, indicating *P. freudenreichii* lactate consumption rate could keep up with the lactate

production rate of both *Lb. plantarum* anb *L. cremoris*, except for the initial hours of incubation where minimal lactate presence is detected when *Lb. plantarum* is present in the co-culture. Mono-cultures of *Lb. plantarum* reached around 21 mM lactate after 4 days. Assuming a similar amount of lactate was produced in co-cultures with *P. freudenreichii*, one would expect to find ~14 mM propionate and 7 mM acetate production by *P. freudenreichii* assuming a 2:1 propionate:acetate ratio in anaerobic conditions from complete lactate consumption. We found a production of ~15 mM propionate and 13.7 mM acetate. When assuming *Lb. plantarum* produced similar amounts of acetate in co-culture (8.7 mM), a total formation of 7 mM acetate by *P. freudenreichii* is found. These values nicely match with expected propionate:acetate ratio of 2:1 and expected total formation based on the lactate produced by *Lb. plantarum*. A similar trend for the co-culture with all three species is observed, although part of the expected formed lactate (~4 mM) cannot be accounted for. In co-cultures of *P. freudenreichii* and *L. cremoris* more propionate and acetate is formed than can be accounted for based on mono-cultures of *L. cremoris* and no ethanol formation is observed by *L. cremoris*, indicating at least partial competition for substrates between *P. freudenreichii* and *L. cremoris*. All in all these results do not indicate any negative effect of *P. freudenreichii* on metabolite production by *Lb. plantarum* and hence it is unlikely *P. freudenreichii* competes for carbon sources with *Lb. plantarum*. For interactions with *L. cremoris* at least some indication for substrate competition exists. This could be due to slower and less lactate release by *L. cremoris* compared to *Lb. plantarum*, making *P. freudenreichii* compete for sugars instead of relying mainly on lactate present in its enviroment (discussed below). It is plausible that, although being able to grow on and metabolize present sugars in lupin milk, *P. freudenreichii* prefers lactate whenever it is present and does not compete with the lactate-producer in its microbial consortium when there is sufficient supply of lactate. This hypothesis is supported by the fact that indeed a preference of lactate over glucose is reported for *P. freudenreichii* when both carbon sources are present (Lee et al. 1974).

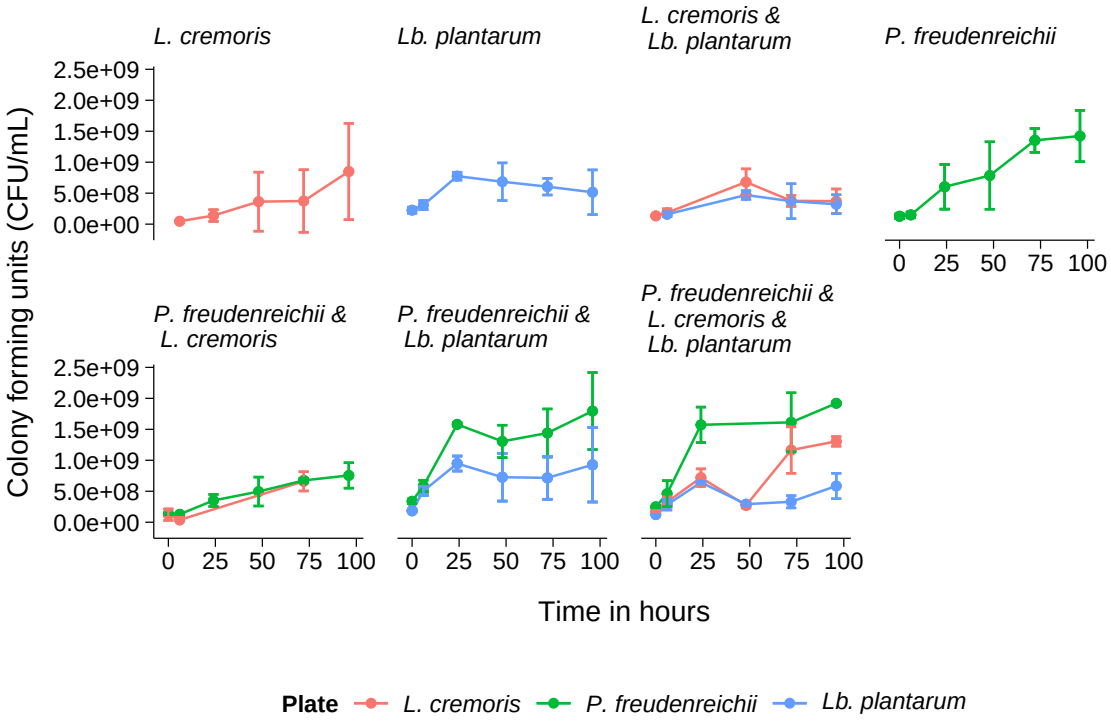

Figure 6.3: Bacterial growth during lupin quark production

Bacterial growth during lupin quark production

Lupin milk was found to support growth of all three bacteria in mono-culture (see Figure 6.3). *P. freudenreichii* was able to ferment lupin milk by itself reaching cell densities of $1.4 * 10^9$ cfu/mL, which was higher compared to *L. cremoris* ($8.5 * 10^8$ cfu/mL) and *Lb. plantarum* ($5.2 * 10^8$ cfu/mL). In co-cultures, both LAB seem to compete with each other as after 24h in co-culture with *L. cremoris*, *Lb. plantarum* cell numbers dropped below detection limit used in our study (10^6) only to recover later and *L. cremoris* cell numbers also decline to $3.7 * 10^8$ cfu/mL. Similarly, in combination with *P. freudenreichii*, *L. cremoris* was not detected at various time points indicating reduced growth. Interestingly, also *P. freudenreichii* cell numbers declined to $7.5 * 10^8$ cfu/mL.

The effects observed in co-culture between *L. cremoris* and *P. freudenreichii* or *Lb. plantarum* are not evident in co-culture with all three bacteria. Therefore the most plausible explanation for the drop in cell numbers of *P. freudenreichii* when grown solely together with *L. cremoris* seems to be competition for substrates,

as *L. cremoris* is able to utilize the same carbon sources as *P. freudenreichii*, but releases less and more slowly lactate to the lupin milk compared to *Lb. plantarum*, in which co-cultures reduced *P. freudenreichii* growth is not observed. This reduced release of lactate may trigger *P. freudenreichii* to compete for carbon sources, as lactate release by *L. cremoris* may be below *P. freudenreichii* requirements. This hypothesis would be supported by the fact that at 6 hours no lactate can be detected in co-cultures of *P. freudenreichii* and *L. cremoris*, whereas in co-cultures with *Lb. plantarum* a minimum lactate presence is detected (see Figure 6.2). The release of lactate by *Lb. plantarum* seems to increase growth rate of *P. freudenreichii* compared to mono-cultures of *P. freudenreichii*, as higher cell numbers are found after 1 day. Growth of *Lb. plantarum* was not affected in co-culture with *P. freudenreichii*, indicating indeed *P. freudenreichii* did not compete for carbon sources with *Lb. plantarum*. In general, *P. freudenreichii* is applicable in co-cultures with *Lb. plantarum*, whereas the combination with *L. cremoris* in our study is less promising. It would be interesting to see if similar growth behavior of *P. freudenreichii* and *L. cremoris* occurs when a small amount of lactate is added (~10 mM) at the initial stages of fermentation, thereby elucidating whether our observations are a result of substrate competition between the two microbes.

Free amino acid profiles of lupin quark

Free amino acid content was determined during lupin quark fermentation for 18 free amino acids. Notably, initial contents and free amino acid profile developments were variable between samples, showing biological variability between different batches of lupin beans processed into milk and as a result differences during lupin milk fermentation. For each mono-culture it was determined whether free amino acids were present in higher or lower amounts after 96 hours compared to 0 hours. It must be noted higher free amino acids amounts can occur either due to liberation from peptides or proteins, whilst *de novo* synthesis and export can also not be excluded. Due to the high variability, as rule of thumb for potential amino acid consumption or release a 20% deviation from the initial start point value was taken (see Table 6.1).

Table 6.1: Amino acid release or consumption per species. (+)-sign indicates >20 percent release, (+/-)-sign indicates <=20 percent difference from starting concentrations, (-)-sign indicates >20 percent consumed during fermentation.

	L. cremoris	*Lb. plantarum*	*P. freudenreichii*
Alanine	(+)	(+)	(+)
Aspartic acid	(+)	(+)	(-)
Glutamic acid	(+)	(+)	(+)
Glutamine Arginine	(-)	(+/-)	(-)
Glycine	(+)	(+)	(+/-)
Histidine	(+)	(+)	(-)
Isoleucine	(+)	(+)	(+)
Leucine	(+)	(+)	(+)
Lysine	(+/-)	(+)	(+)
Methionine	(+)	(-)	(+)
NH3	(+)	(+)	(+)
Phenylalanine	(+)	(+)	(+)
Proline	(+)	(+)	(+)
Serine	(-)	(-)	(-)
Threonine	(+/-)	(+/-)	(+/-)
Tryptophan	(+)	(+/-)	(+)
Tyrosine	(+)	(+)	(+)
Valine	(+)	(+/-)	(+)

Out of 18 free amino acids analyzed, 7 amino acids were released by all three bacteria during the fermentation (glutamate, alanine, proline, tyrosine, isoleucine, leucine and phenylalanine), see Appendix Figures 6.14, 6.15, 6.16. Furthermore all mono-culture samples contained higher amounts of NH_3 at 96 hours, indicating ammonia release from amino acid catabolism by all species (Vince and Burridge 1980).

For all three species significant release of free amino acids was observed (Figure 6.4), indicating some form of proteolytic or peptidase activity. Serine was found to be the sole amino acid consumed by all species and was depleted completely by all species at t=96 hours. Serine can be deaminated to pyruvate and can act as significant contributor to total fermentation end-products in *L. lactis* (Aller et al. 2014; Novak and Loubiere 2000) and *Lb. plantarum* (Liu et al. 2003) and likely is utilized as source of carbon by *P. freudenreichii* (Crow 1987) as well. As reported previously, aspartate was consumed by *P. freudenreichii* and likely converted to fumarate by aspartate:ammonia lyase (Falentin et al. 2010) to act as additional

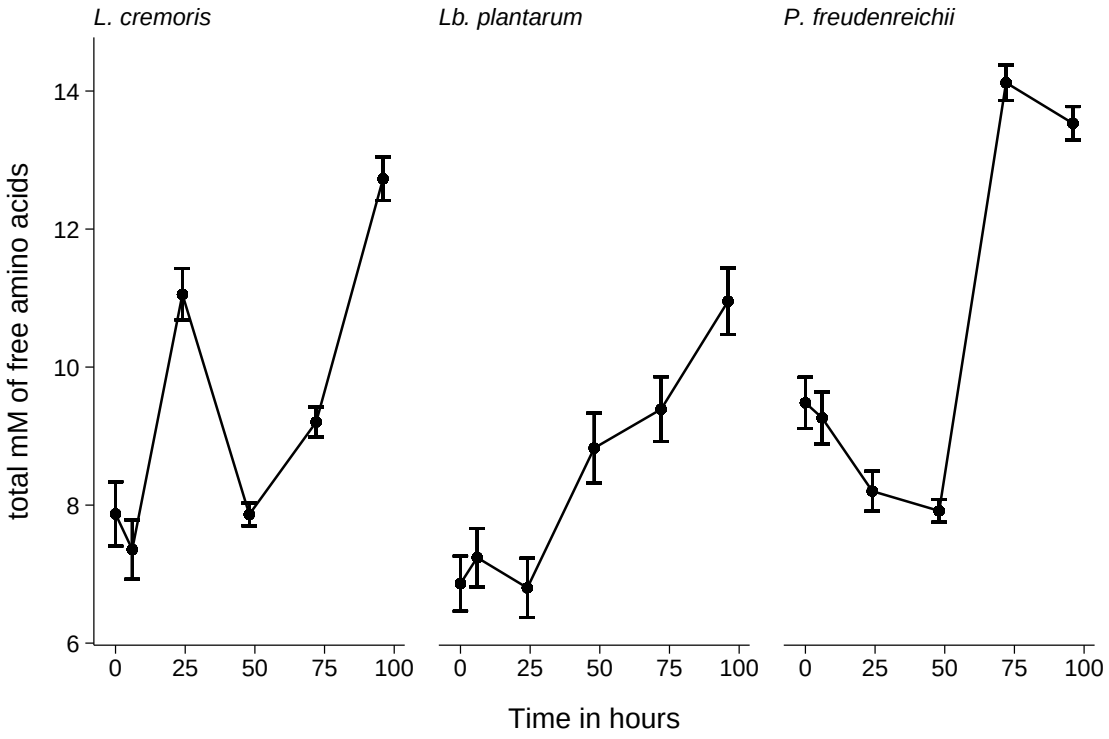

Figure 6.4: Total free amino acid content during lupin quark production by fermentation of *L. cremoris*, *Lb. plantarum* and *P. freudenreichii*

electron acceptor (Conway 2021). Next to aspartate and serine, also consumption of glutamine/arginine was observed for *P. freudenreichii*, in line with earlier reports (Dalmasso et al. 2012; Gagnaire et al. 2015; Gagnaire et al. 2001). *L. cremoris* was found to have similar or higher contents of free amino acids for 16 out of 18 amino acids. Next to serine, also utilization of glutamine was observed. Glutamine acts as one of the most important media constituents for high biomass yields and is essential for growth in environments with low amino acid contents (Aller et al. 2014). Glutamine act as nitrogen donor in *L. lactis* and can be converted to glutamate and proline (Larsen et al. 2006).

To evaluate whether specific bacteria could be correlated with specific free amino acid abundances in co-cultures, a heatmap (Figure 6.5) was constructed based on relative abundance (Z-scores, see materials and methods section). Mono-cultures of *L. cremoris* were associated with the highest relative abundance of 16 out of 18 free amino acids. Only glutamine/arginine and lysine abundance was found below

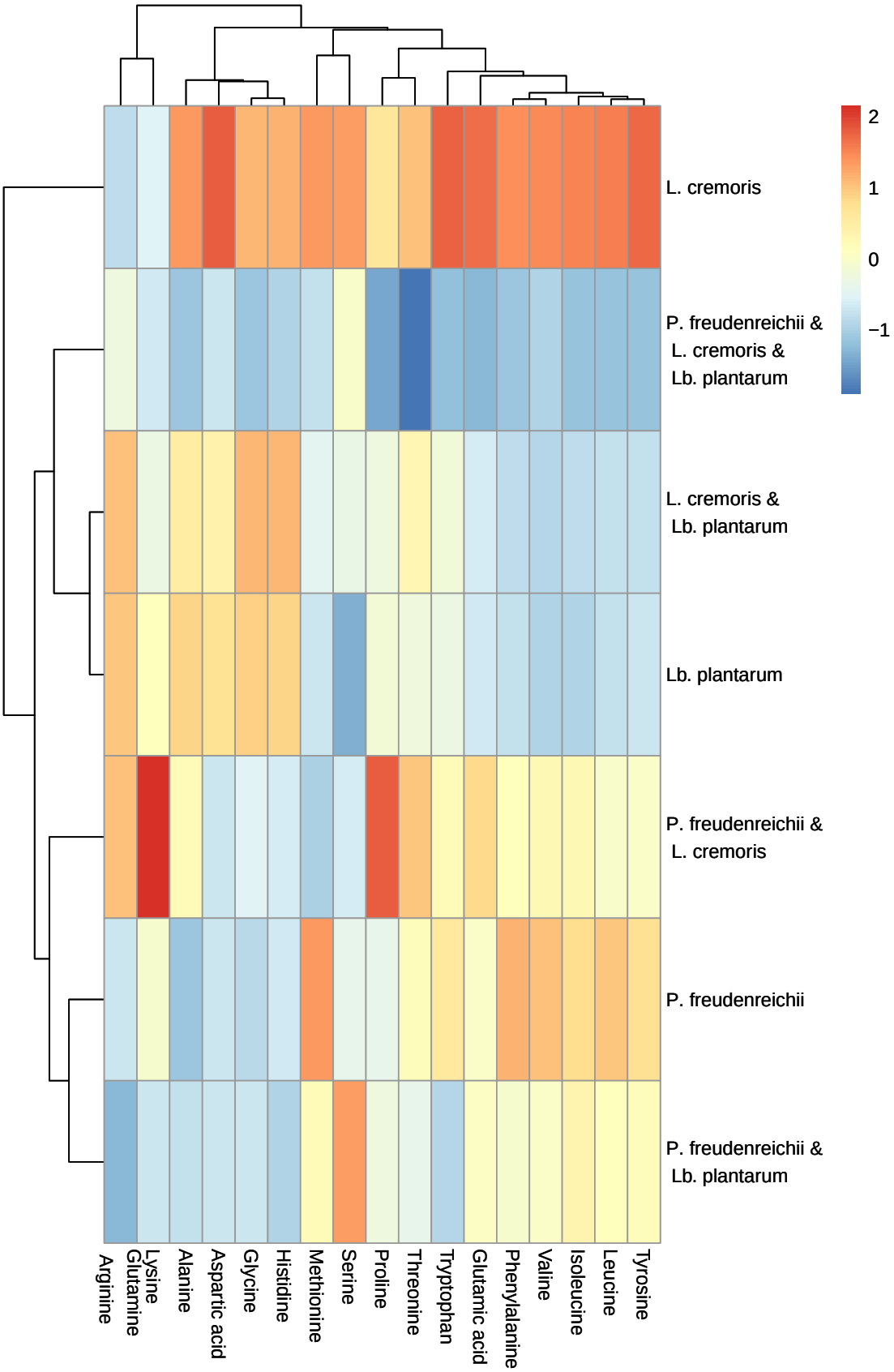

Figure 6.5: Relative abundance of amino acid contents in lupin quark after 96 hours. Negative values (blue) indicate relative low abundance, Positive values (red) indicate relative high abundance.

average amongst samples. Arginine and lysine are major constituents of *L. lactis* cell walls (Driessen et al. 1989) and hence required for growth. Low abundance of alanine, aspartic acid, glycine and histidine were associated with fermentations containing *P. freudenreichii*. Likewise, fermentations containing *Lb. plantarum* were associated with low abundance of proline, threonine, trpytophan, glutamic acid, phenylalanine, valine, isoleucine, leucine and tyrosine. *Lb. plantarum* WCFS1 lacks biosynthethic pathways for valine, leucine and isoleucine (Kleerebezem et al. 2003) and hence require these amino acids from their environment. Furthermore, for other *Lb. plantarum* strains glutamic acid and threonine were also found to be esssential and phenylalanine and tryptophan stimulatory for growth (Saguir and Nadra 2007). Bacterial requirements for specific amino acids is thus reflected in the final free amino acid content of lupin quark. The increased content of most amino acids indicates there is no bacterial growth limitation in lupin quark due to amino acid limitations.

Vitamin B_{12} production in lupin quark

Fermented lupin milk was assayed for the presence of active vitamin B_{12} by reversed phase High performance liquid chromatography (RP-HPLC). After 4 days of fermentation samples containing *P. freudenreichii* in mono-culture contained 2.9± 0.05 μg B_{12}, *P. freudenreichii* and *Lb. plantarum* 3.1± 0.1 μg B_{12} and *P. freudenreichii*, *Lb. plantarum* and *L. cremoris* 2.8± 0.04 μg B_{12} per 100 gram of fermented product (see Table 6.2).

Interestingly, production of B_{12} was thus highest in the co-culture of *P. freudenreichii* and *Lb. plantarum* compared to the mono-culture of *P. freudenreichii* or

Table 6.2: Lupin quark B_{12} contents at 96 hours.

Sample	B_{12} content (μg/100 gram product)
P. freudenreichii	2.9 ±0.05
P. freudenreichii, Lb. plantarum	3.1 ±0.10
P. freudenreichii, Lb. plantarum, L. cremoris	2.8 ±0.04
L. cremoris	0.0 ±0.00
Lb. plantarum, L. cremoris	0.0 ±0.04
Lb. plantarum	0.0 ±0.00

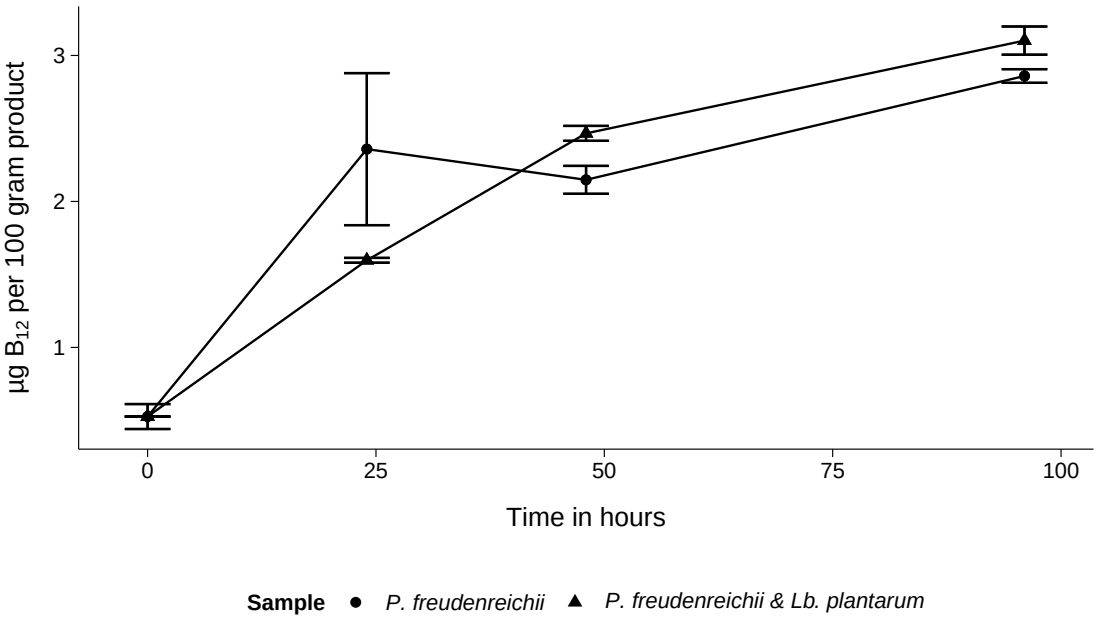

Figure 6.6: B_{12} formation in time in lupin quark produced by *P. freudenreichii* or *P. freudenreichii* and *Lb. plantarum*

co-culture containing all three bacteria ($p<0.05$, tukey HSD), albeit at minimum differences. The production of B_{12} after 4 days per 100 gram product in all samples more than meets the daily suggested intake of 2.4 μg per day. To monitor production speed, additional B_{12} measurements of lupin milk fermented with solely *P. freudenreichii* and with *P. freudenreichii* and *Lb. plantarum* were taken at 24 and 48h (see Figure 6.6). After 24h samples containing only *P. freudenreichii* contained 2.4± 0.5 μg B_{12} per 100 g and samples containing *P. freudenreichii* and *Lb. plantarum* contained 1.6± 0.2 μg B_{12} per 100 g. Considerable amounts of B_{12} are thus produced on lupin milk within a day. The obtained yield on lupin is in the same range as found by Wolkers–Rooijackers et al. (2018), who observed a yield of 0.97 μg B_{12} per 100 g lupin tempeh produced with *P. freudenreichii*. Lupin, other legumes and cereals allow for significant *in situ* B_{12} production by *P. freudenreichii* (Xie et al. 2021). *P. freudenreichii* is therefore a promising candidate for *in situ* B_{12} enrichment of plant-based foods.

Conclusion lupin quark

In general, *P. freudenreichii* was able to ferment lupin milk in mono or in co-culture. The presence of *P. freudenreichii* affects organic acids profiles, as not lactate but propionate and higher amounts of acetate can be found. Results indicate *P. freudenreichii* only competes for sugar carbon sources when there is no sufficient release of lactate by consortium members. The final product had a higher pH, probably due to consumption of lactate by *P. freudenreichii*. Free amino acid data indicates free amino acid contents increase during fermentation and hence do not pose as growth-limiting factor for any of the bacteria. All obtained products fermented with *P. freudenreichii* contained vitamin B_{12} in high amounts, surpassing the daily suggested intake by consumption of 100 grams of fermented product already. *P. freudenreichii* thus has proven to be an interesting microbe for *in situ* B_{12} fortification of fermented food products, exemplified by our lupin milk fermentation.

Enhancing product characteristics of fermented foods by cross-over fermentation: The dairy miso case study

Cross-over fermentations can besides nutritional enrichment also be used for creating novel fermented food products or enhancing existing product characteristics. Below, an example by Dank, Mastrigt, Yang, et al. (2021) is discussed in which miso, a traditional japanese fermented soybean paste, was combined with quark, unripened cheese commonly produced in Europe from cow's milk, to produce a novel food product called dairy miso.

Quark is made from heat-treated milk which is inoculated with starter lactic acid bacteria, usually *Lactococcus lactis* and in some cases rennet, resulting in acidification to a pH of ~4.5 and gelation. Traditionally, the whey is removed from the curd using linen cloth bags, whereas in industrial processes this is usually replaced by mechanical methods. The result of this process is a smooth creamy white product with a fresh and mildly acidic taste (Farkye 2017).

Miso is traditionally produced by fermenting soybeans with *A. oryzae* pre-cultured on rice (called koji) in the presence of salt contents ranging from 55 to 200 g salt/kg product for up to 3 years (Shibasaki and Hessbltine 1962). In addition to soybeans alone, many variations in grain and bean substitutes can be used for making miso (Shurtleff and Aoyagi 1979a). Koji is made from cooked polished rice grains inoculated with *A. oryzae* incubated at 30 to 35° C for 2-3 days whilst regularly mixing. Before the mould starts sporulation, it is inoculated on to the soybeans (Shurtleff and Aoyagi 1979b). *A. oryzae* has been used for solid-state fermentations already since 3000-2000 years ago in China and has a long history of use since 700 B.C in Japan for production of soy sauce, Japanese spirit (shochu), Japanese rice wine (sake) and miso (Gomi et al. 2007). *A. oryzae* is known to secrete many hydrolytic enzymes during solid state fermentations (Machida et al. 2008), like lipases (Ohnishi et al. 1994), proteases and amylolytic enzymes (Maeda et al. 2004). Furthermore, *A. oryzae* has been shown to produce $\beta-$galactosidase (Akasaki et al. 1976). These characteristics make *A. oryzae* a potential candidate for fermentation of dairy substrates.

Dairy miso resembles sweet-scented blue mould cheese

Dairy miso was produced by inoculating koji (*A. oryzae* grown on rice) in quark in a 1:3 (weight/weight) ratio using 60 g/kg NaCl, a salt concentration used in production of red sweet miso (Shurtleff and Aoyagi 1979a). Consequently, the aroma formation (volatile organic compounds, VOCs) over time was monitored by frequent sampling. VOC content increased drastically over time, see Figure 6.7. A total of 77 individual components were detected, in which the aroma profile was largely dominated by acids and ethyl esters thereof (file A.1, supplementary data).

The profiles of VOCs detected in the dairy miso were compared with those of commercial blue mould cheese and white mould cheese. The total amount of VOCs found in dairy miso exceeds the amount detected on blue and white mould cheeses with up to 3 times more total response (Figure 6.8). Compared to mould cheeses a relatively high amount of higher alcohols and esters was found in dairy miso,

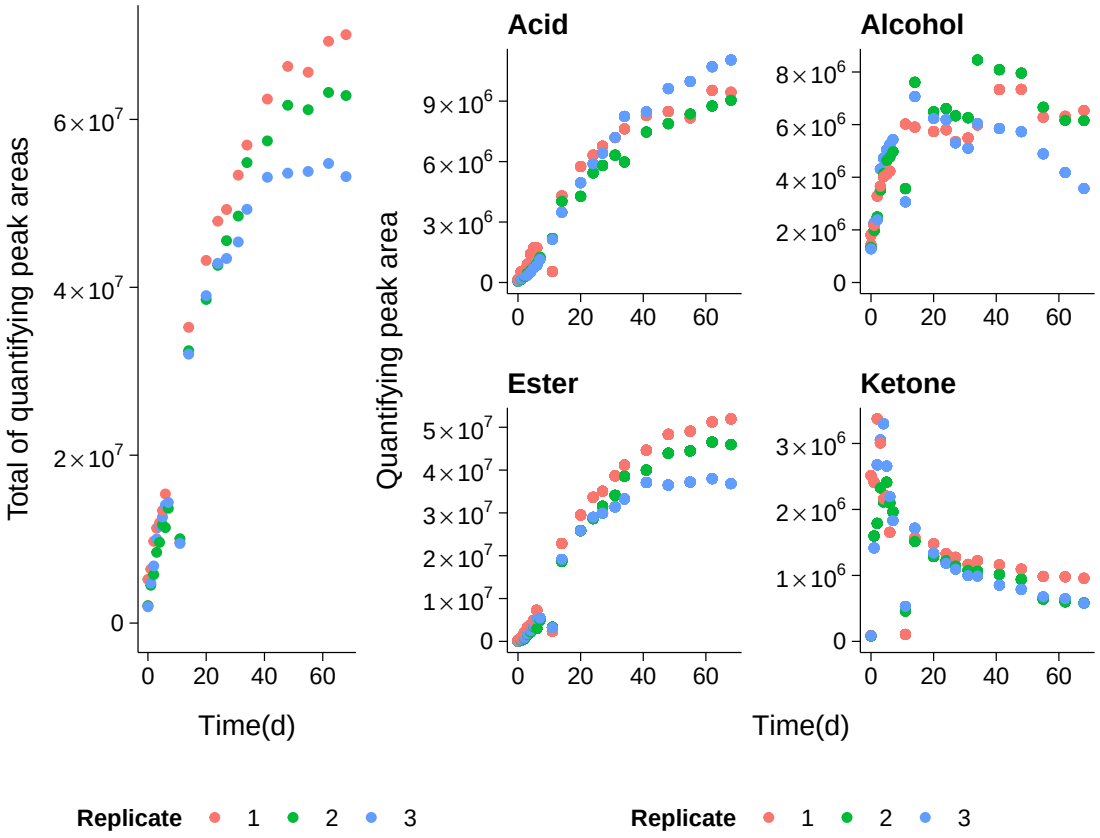

Figure 6.7: Volatile organic compound development in dairy-based miso produced with 60 g/kg NaCl and *A. oryzae*. The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group at each time point is shown on the right. Aroma formation was followed over a course of 68 days. Biological replicates (n=3) are displayed by different colours.

resulting in very strong sweet and floral notes and less pungent ketone smell typical for mould cheeses (Spinnler and Gripon 2004). These sweet notes at lower salt contents are due to the production of fruity ethyl esters (i.e. ethyl pentanoate(apple), ethyl hexanoate(pineapple), ethyl heptanoate(fruit) and many others, see Figure A.1, supplementary data) derived from fatty acid metabolism and ethanol. In order to form ethyl esters in (dairy) miso, ethanol and acyl-CoA, which derive from free fatty acids, are required together with alcohol O-acetyltransferase activity (Saerens et al. 2006). Thus, both ethanol (primary metabolism) and free fatty acid concentrations (lipolytic activity) may be the rate determining factors for final ethyl ester content found in dairy miso.

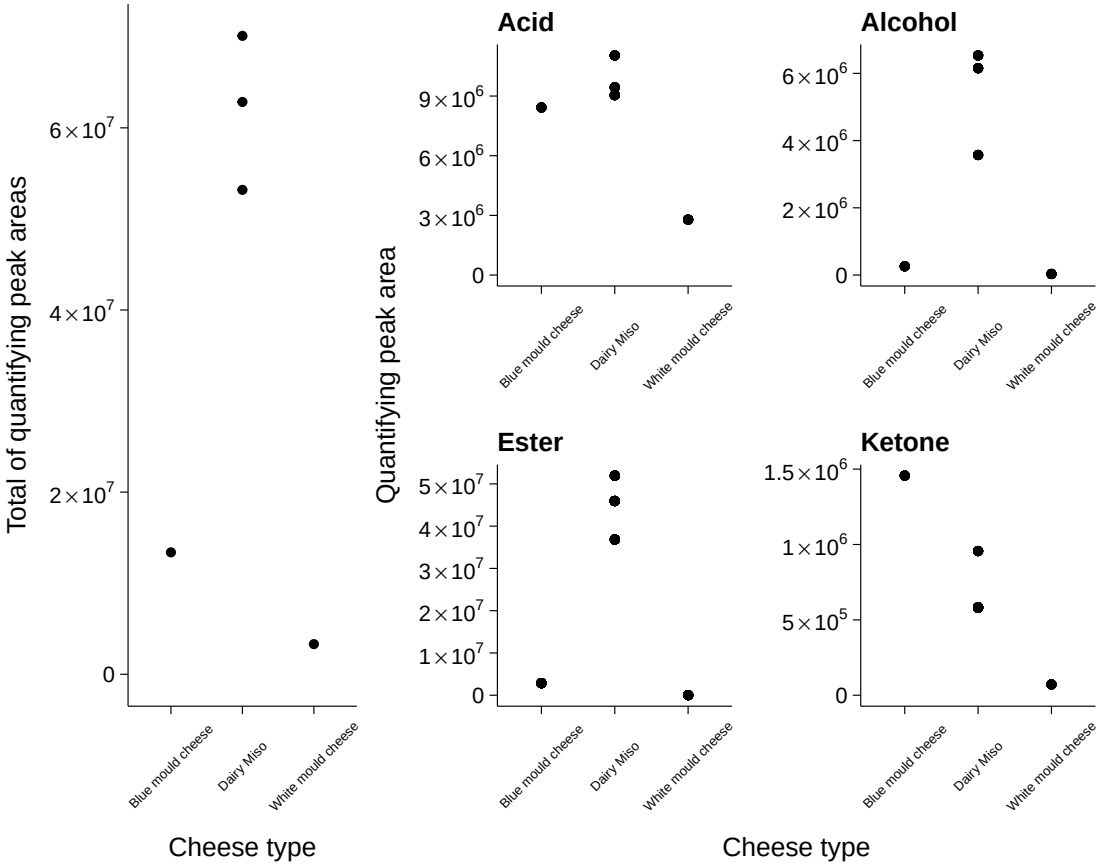

Figure 6.8: Comparison of volatile organic compound contents between a commercial blue mould cheese, white mould cheese and dairy miso of 68 days old produced with 60 g/kg NaCl and *A. oryzae*. The total volatile organic compound development is shown on the left. Each volatile organic compound was assigned to a compound type (acids, alcohols, esters and ketones) and summed. The total of each group is shown on the right.

Traditional production practices influence final product characteristics

Salt content affects lipolytic activity of *A. oryzae*

In traditional miso production various sodium chloride contents are used to produce different kinds of miso flavours (Shurtleff and Aoyagi 1979a). Addition of salt lowers the water activity (a_w) of products and correspondingly affects the fermenting microorganisms. Generally, traditional miso with a low salt content (<70 g/kg) tends to ferment quickly, resulting in a miso with a sweet taste, whilst miso with a higher salt content (>100 g/kg) has a more savoury taste (Shurtleff and Aoyagi 1979a).

Dairy miso produced with NaCl contents ranging from 0 to 200 g/kg show *A. oryzae* is not affected at large in terms of aroma production up to 80 g/kg NaCl and

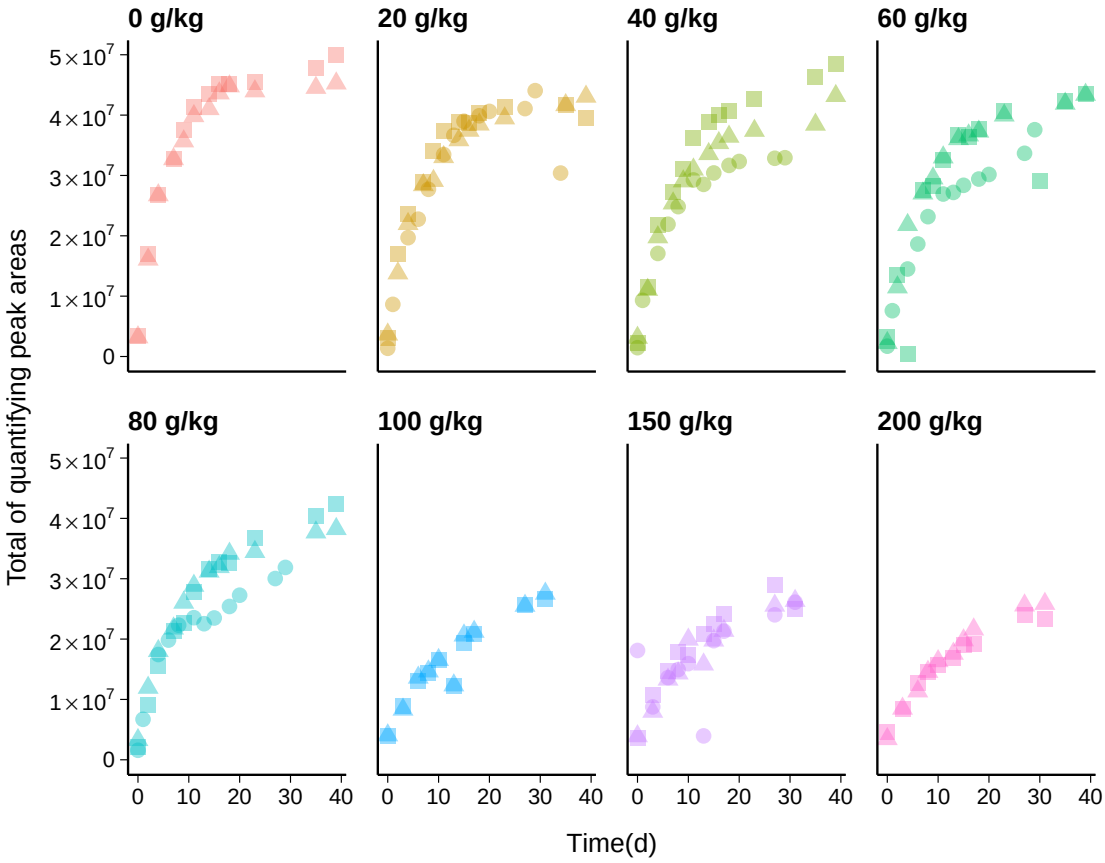

Figure 6.9: Total volatile organic compound development for dairy miso produced using NaCl contents ranging from 0 to 200 g/kg over a course of 30 days. Biological replicates (n=3) are displayed by different shapes.

is able to produce significant amounts of aroma up to 200 g/kg NaCl (Figure 6.9).

However, at =>100 g/kg salt significant lower aroma production rates are found compared to <80 g/kg salt containing dairy miso (Figure 6.10, file A.2, supplementary data) signifying a reduced metabolic capacity. Indeed visual observations showed more viscous samples above 100 g/kg salt containing dairy miso, indicating reduced proteolytic and/or lipolytic activity, an observation which is also made in blue cheese in which the addition of salt reduces proteolytic activity of *Penicillium roqueforti* (Kinsella et al. 1976).

Lower free fatty acids and ester contents (Figure 6.11) and production rates (file A.2, supplementary data) were found, pointing towards reduced lipolytic activity of *A. oryzae*. Primary metabolism of lactose was not affected at large between the different salt concentrations and the main metabolite produced remained ethanol

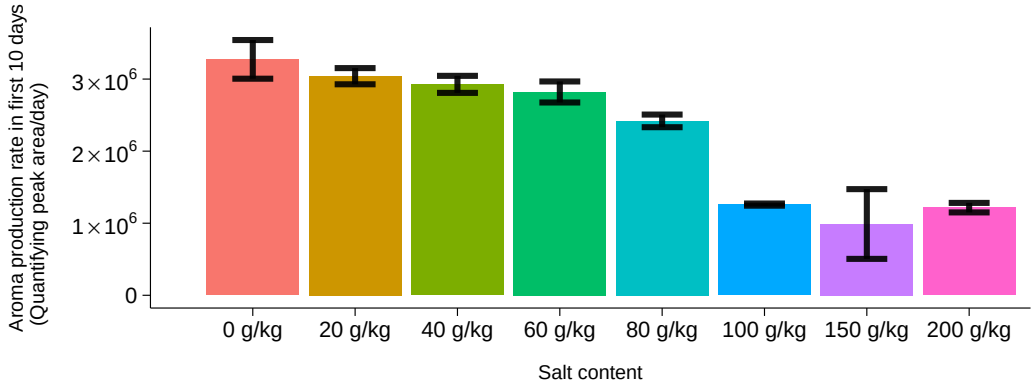

Figure 6.10: Volatile organic compound production rates for different NaCl contents estimated by fitting linear models for the first 10 days for biological replicates (0 to 80 g/kg NaCl n=3, 100,150,200 g/kg NaCl n=2). Slopes of the linear models were considered to be the aroma production rate per day.

(Figure 6.12). Primary metabolism of sugars was therefore not affected to large extends by increments of salt. Indeed, *A. oryzae* has been shown to be able to grow at an a_w of 0.85 (Gibson et al. 1994), whereas dairy miso with a salt content of 200 g/kg had an a_w of 0.861.

Next to ethanol also pyruvate, acetate, citrate, butyrate, glycerol, and propionate were detected (Figure 6.12). Ethanol, pyruvate and citrate production was not notably affected by higher salt contents, agreeing with no noticable decline of glycolytic activity. Butyrate, a common milk fat hydrolysis product in blue cheese (Kinsella et al. 1976), glycerol a product of fat hydrolysis (Fu et al. 1995) and propionate (Figure 6.12, Figure A.2, supplementary data) production decreased at high salt contents. This is in agreement with the maximal lipolytic activity in blue mould cheese, which occurs between 40-60 g/kg NaCl (Fox et al. 2004). These results clearly show fat hydrolysis slowed down by the addition of salt. It seems therefore that the main effect of salt is a decreased lipolytic activity, whereas glycolytic activity is not affected at large.

Initial fat content determines volatile organic compound composition

The dominant VOCs found in dairy miso produced with full fat quark mainly originate from fat degradation. Indeed, dairy miso produced with quark with a

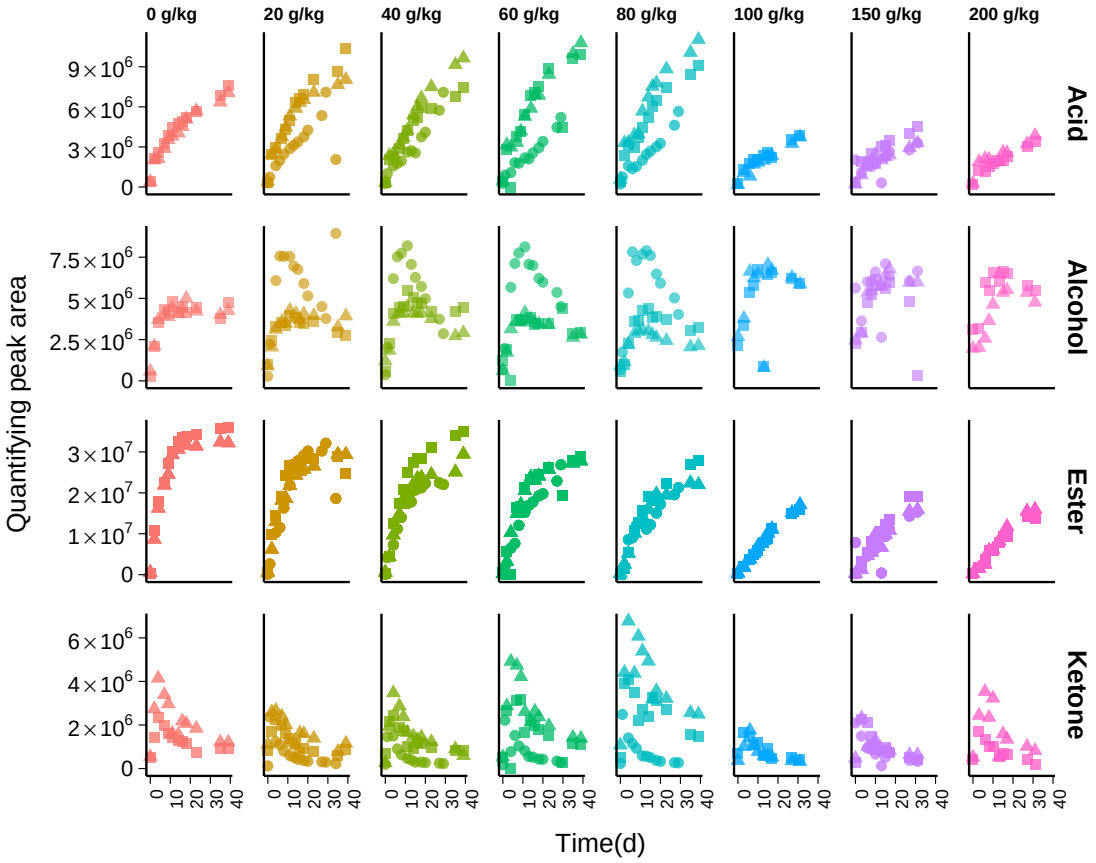

Figure 6.11: Volatile organic compound development per compound type for dairy miso produced using NaCl contents ranging from 0 to 200 g/kg over a course of 30 days. Biological replicates are displayed by different shapes (0 to 80 g/kg NaCl n=3, 100,150,200 g/kg NaCl n=2).

low fat content (0.5 g/100g) had lower ester contents compared to full fat quark (8.6 g/100g). Products of fat hydrolysis all declined drastically (Figure 6.13), clearly demonstrating the importance of milk fat hydrolysis in aroma formation of dairy miso and demonstrating the importance of soy bean oil for traditional miso. Accordingly, in blue mould cheeses lipase activity of the moulds is responsible for characteristic aroma due to liberation of free fatty acids and subsequent degradation into ketones (Spinnler and Gripon 2004). Notably, higher alcohol contents were found in low-fat quark compared to full-fat quark, showing alcohol formation during dairy miso production mainly originates from carbohydrate substrates present in the quark and rice, i.e. lactose and glucose.

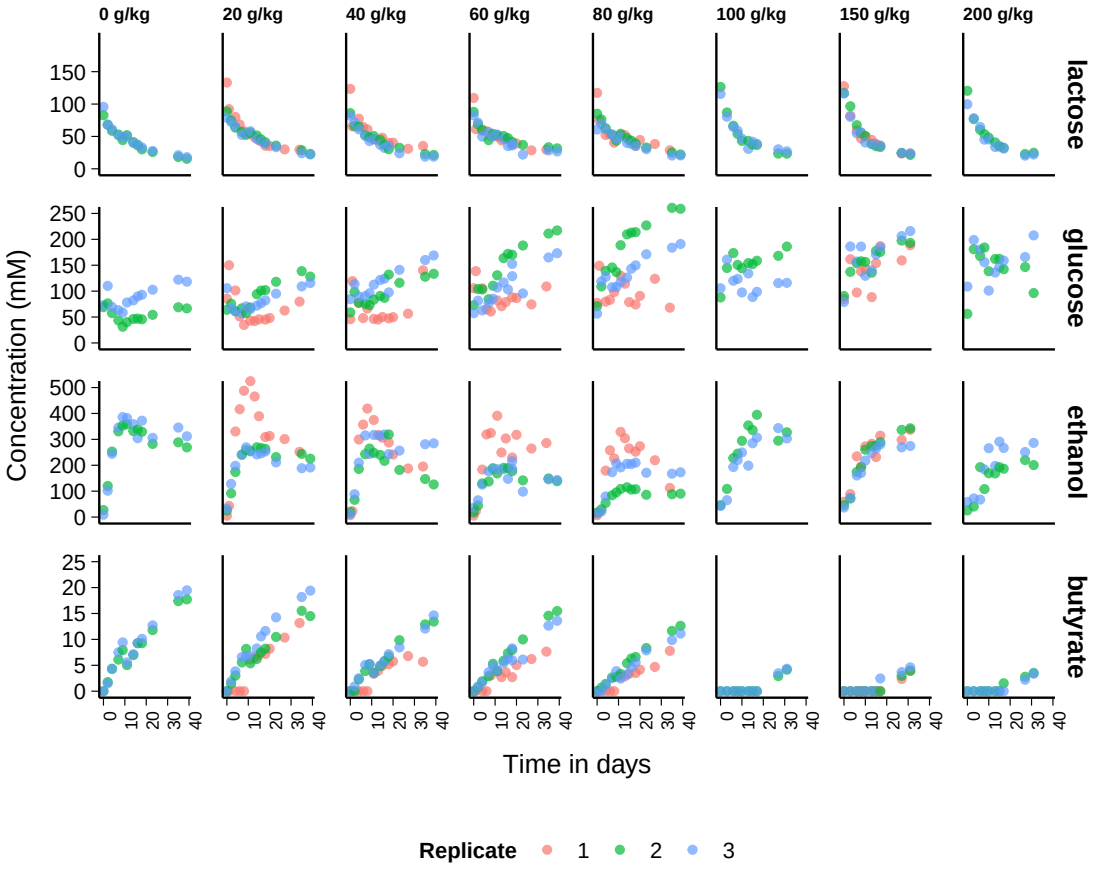

Figure 6.12: Metabolite consumption and production during dairy miso production using various NaCl contents and *A. oryzae*. Each miso was followed over a time period of at least 30 days. Biological replicates (n=3) are displayed by different colours.

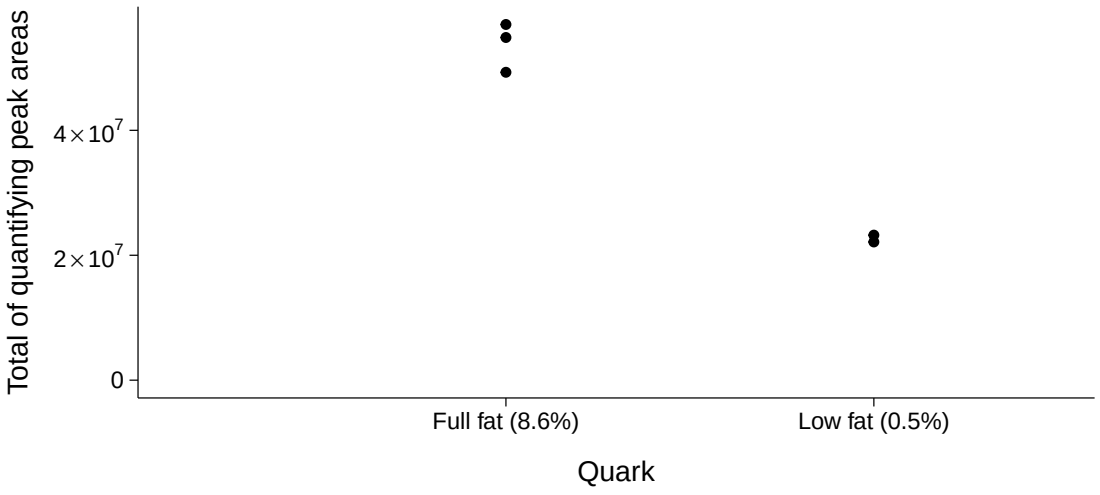

Figure 6.13: Effect of initial fat content on volatile organic compound formation of dairy miso after 28 days using 60 g/kg NaCl (n=3 for full-fat, n=2 for low-fat quark).

Conclusion dairy miso

In general, by using tradition process parameters like salt and fat content it is possible to steer volatile organic compound formation in dairy miso. Targeting both the fungal primary metabolism or extracellular fat hydrolysis by changing these parameters gives an opportunity to steer formation of specific compound groups, resulting in more sweet/floral or savory dairy miso. The use of *A. oryzae* for fermentation of quark resulted in a novel food product with interesting characteristics, which exemplifies the possibilties for novel fermented food product development. Traditional process parameters can be used to affect the fermenting organism during cross-over fermentations, resulting the possibility to steer product characteristics during novel fermented food product development.

General discussion

Interesting spontaneous fermentations without addition of starter cultures are carried out all across the globe. The microorganisms in these fermentations convert raw material into fermented food products and thereby can increase shelf-life, nutritional value and organoleptic properties (Nout et al. 2007). Manufacturer's practices such as back-slopping, temperature regimes and fermentation duration shapes microbial communities (Moonga et al. 2021; Moonga et al. 2020) and re-using fermentation equipment decreases microbial diversity over time, as the fermentation will be driven more and more by the dominant species (Groenenboom et al. 2020). Historically speaking this has led to isolation of these dominant species and the utilization of single-strain or multiple-strain defined starter cultures. A clear benefit of isolation of these dominant players is that they are adapted to fermentation of the raw material. Furthermore, the use of defined starters increases product consistency and safety (Ross et al. 2002). In addition, if strains have been historically used for a long time they have evolved (are domesticated) to be better adapted to utilization of the raw material in which they are applied. Dairy *L. lactis* strains for instance have acquired genetic material that facilitates more efficient use of milk (Bachmann et al. 2012;

Kelly et al. 2010). Likewise, *Saccharomyces cerevisiae* can be clustered genetically based on its biotechnological application (Legras et al. 2007) and domestication of *S. cerevisiae* in beer brewing has led to drastic changes in genome structure and stability (Gallone et al. 2018). Domestication leads to strains with desirable fermentation properties and to strains that have been better adapted to the raw material, leading to faster production processes. Furthermore, domestication of microbes can lead to the loss of undesirable traits, such as production of toxins (Gibbons and Rinker 2015; Gibbons et al. 2012). However, a clear drawback is that the metabolic activity displayed during fermentations using defined starters is limited to the genetic potential of the applied starter culture. Often, domesticated starters have smaller chromosomes due to genomic decay (Gibbons and Rinker 2015; Gibbons et al. 2012; Kelly et al. 2010) and therefore are less flexible for application in other raw material. Furthermore, utilization of defined starters instead of spontaneous fermentation could reduce nutritional quality of a product, such as exclusion of B_{12} producing *Klebsiella pneumoniae* or *Citrobacter freundii*, which are not associated with the starter mould, (Keuth and Bisping 1994; Nout et al. 2005) in tempeh.

Although often desirable and beneficial traits are displayed by defined-starters in production of conventional fermented food products, the genetic drawback may pose a limitation in novel food product development. Indeed, diversification of microbial communities in food fermentation is associated with more intense food flavor (Gänzle 2022). For instance, in cheese production non-starter lactic acid bacteria significantly contribute to aroma formation (Lo et al. 2018), even if they are in low abundance (Mastrigt et al. 2019); sourdough bread (spontaneous fermentation) has higher aromatic complexity and intensity compared to bread made with bakers yeast (Hansen and Schieberle 2005) and Chinese liquor *Baijou*, which is microbially very diverse, has more organoleptic complexity than whiskey which is microbially less diverse (Gänzle 2022). When designing novel products single strain genomic scarcity of domesticated strains thus may pose a hurdle. This is exemplified by an attempt to brew low-alcoholic 'fruity' beer. The high ethanol-forming nature of *S. cerevisiae* in its relation to release of 'fruity' volatile organic

acids limits its application in the development of 'fruity' low-alcoholic beers (ignoring technological solutions) (Rijswijck et al. 2017). In contrast, application of non-domesticated non-conventional yeasts *Cyberlindnera fabianii* and *Pichia kudriavzevii* isolated from masau fruits from Zimbabwe (Nyanga et al. 2007) did result in fruity low-alcoholic beers (Rijswijck et al. 2017). The use of non-conventional yeasts genera such as *Dekkera*, *Hanseniaspora*, *Pichia*, *Wickerhamomyces* and others in beer brewing shows potential for development of novel craft beers with desirable properties such as reduced calory and alcohol contents or for bioflavoring (Basso et al. 2016). Since in modern day era we have the scientific knowledge to construct which microbes in a microbial consortium may display the beneficial activity we may use these microbes in the design of novel fermented food products, aiming at increasing desirable organoleptic or nutritional properties. The vast microbial diversity out there in spontaneous fermentations carried out all across the globe provides ample opportunities at identifying and applying microbes with beneficial traits in non-conventional novel food fermentations.

Conclusion

Cross-over fermentations are processes in which a microorganism from one traditional fermentation process is introduced onto a new substrate and/or to a new partner (Dank, Mastrigt, Yang, et al. 2021). Here we show the potential of cross-over fermentations in nutritionally enriching food products or development of novel fermented foods. We demonstrated *P. freudenreichii* is an excellent candidate for *in situ* enrichment of B_{12} in food products. Its low nutritional requirements (Falentin et al. 2010; McCubbin et al. 2020) and preference of lactate over sugars (Lee et al. 1974) make it an excellent microorganism for inclusion in defined starter cultures. Cross-over fermentations also show a large potential in the development of novel food products, as exemplified by the dairy miso study. We showed interesting novel food products can be designed and taking into account traditional production parameters novel fermented food product characteristics can be steered. Next to enhanced nutritional quality or interesting organoleptic properties, cross-over

fermentations may also be used to utilize locally grown substrates. The use of locally grown substrates instead of imported substrates, such as replacing soy with a native European bean such as lupin, can result in more sustainable food production (Wolkers–Rooijackers et al. 2018) by reducing food mileages. Nutritional enrichments by fermentation with vitamin producers also enables dietary switches towards more plant-based protein sources, thereby showing great potential in applications aiding the protein transition from animal towards plant-derived protein sources which is needed to feed the world in the future (Aiking and Boer 2018). The enormous diversity of microorganisms used in traditional fermentation processes and the vast number of alternative substrates offer numerous opportunities for the development of novel fermented products.

Declaration of interest

Declarations of interest: none.

Acknowledgements

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Materials and methods

Lupin quark

All Materials and methods (except for B_{12} analysis, bacterial growth counts and free amino acid data analysis, see below) for lupin quark study were identical to the methods used by Canoy (2021) and are available upon request. Below a brief description of all methods is found.

Strains and pre-culture media

P. freudenreichii DSM 20271 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and routinely grown on Yeast extract lactate broth and agar with composition described in Dank, Mastrigt, Boeren,

et al. (2021) in anaerobic conditions. *Lb. plantarum* TMW 1.460 was obtained from Lehrstuhl für Technische Mikrobiologie Weihenstephan (Technical University of Munich, Freising, Germany) and routinely grown on MRS broth and agar in microaerobic conditions. *L. cremoris* DSM 20388 was obtained from DSMZ and routinely grown on GM17 (Difco M17 supplemented with 0.5% w/v D-glucose) in aerobic conditions.

Lupin milk production

Lupin milk was produced by the methods described by Canoy (2021) with slight modifications. Lupin bits from seeds of *Lupinus albus* were soaked overnight. The soaking water was removed and warm water was added. The mixture was ground for 5 minutes in a food processor (Waring commercial Laboratory blender) and filtered through a four-layered cotton cloth. Lupin milk was pasteurised by heating the milk to boiling point in a microwave (1800 W, 45 seconds) after which it was placed in a 95°C water bath for 60 seconds.

Lupin quark fermentation

Lupin quark was produced according to the methods of Canoy (2021). Greiner tubes were filled with 25 mL of pasteurized lupin milk and inoculated with 1 % of pre-cultures in mono-culture fermentations and in combinations with only 1 lactic acid bacteria. 0.5% inoculum was used for the lactic acid bacteria when both *Lb. plantarum* and *L. cremoris* were present. After inoculation lupin milk was vortexed and incubated at 30°C. Samples were taken at regular time intervals.

Bacterial growth quantification

Bacterial growth in lupin milk was analyzed by serial dilutions of 1 mL of lupin milk in Peptone Physiological Saline solution. *Lb. plantarum* was grown on MRS supplemented with either 20 mM sorbitol (in co-culture experiments with *L. cremoris*) or glucose and incubated in microaerophilic conditions. *Lb. plantarum* was grown on GM17 aerobically. *P. freudenreichii* was grown on YEL with composition described by Dank, Mastrigt, Boeren, et al. (2021) and incubated

anaerobically. *Lb. plantarum* and *L. cremoris* plates were counted after 2 days, *P. freudenreichii* containing plates were counted after 7-10 days. Plates with counts between 10 and 500 were considered valid and cfu/mL was calculated using these counts. If 2 different dilutions were counted within this interval, the average cfu/mL of the two dilutions was used as calculated cfu/mL. Points were only displayed if both replicates at a specific time point were above detection limit (10^6).

Analysis of extracellular organic acids and ethanol

For quantification of extracellular organic acids and ethanol the methods described by Dank, Mastrigt, Boeren, et al. (2021) were used. A standard curve of each component was added to the sequence to quantify each component.

Analysis of sugars

Analysis of sugars was performed according to the methods of Canoy (2021).

Free amino acid quantification and data analysis

Free amino acid profiles were determined by HPLC according to the methods of Scott Jr et al. (2021). A heatmap was constructed using Pheatmap (Kolde and Kolde 2018) in R-studio. Free amino acid content was normalized using Z-scores (Jain et al. 2005), after which hierarchical clustering was performed amongst samples using complete-linkage clustering in Pheatmap (Dank, Mastrigt, Boeren, et al. 2021).

Vitamin B_{12} quantification

Vitamin B_{12} content was determined using the following procedures: Extraction of B_{12} was performed in 100 mL scott flasks containing 35 mL sodium acetate buffer (pH 4.5), 0.5 g Taka-diastase (from *A. oryzae*), 0.2 mL lysozyme (15%, from chicken egg white). Approximatly 10 g of sample was added after which the flasks were incubated in a 30°C waterbath for 30 minutes. Flasks were shaken by hand regularly in-between. After 30 minutes 0.2 mL (1%) Pepsin and 0.25 mL potassium cyanide (4%) was added and samples were placed back for 45 minutes. After 45 minutes, flasks were transferred to a 100°C waterbath for 30 minutes. After

cooling to room temperature, the extract was transferred to 50 mL Greiner tubes and centrifuged at 4°C for 15 minutes. Extracts were filtered through Whatman grade 2v qualitative filter papers (pre-folded, 240 mm) and collected. Extract were then concentrated using Immunoaffinity columns (ds Easy-extract vitamin B_{12}, R-biopharm) into InertSep empty reservoirs (20mL). ~30 mL of filtrate was loaded in the reservoirs and run over the columns, after which columns were washed with 10 mL MiliQ water and flushed dry with a syringe. Next, 4 mL methanol (100%) was loaded over the column and collected in reaction tubes. Tubes were dried at 50°C on a heating block whilst flushing with nitrogen gas after which 1 mL of eluent for LC-MS (10mM ammonium formate + 1 mL formic acid per Liter) was added to the tube, mixed and vortexed. The concentrated extracted B_{12} was filtered through 0.2 μm filters and placed in amber LC-MS vials upon analysis. B_{12} was detected by using Agilent LC-MS with 100mm*3.0mm HSS C18-3.5 μm column at a flow rate of 0.4 mL/min eluent with composition described above and injection volume of 15 μL. An eluent gradient was used with 2 mobile phases. Phase A consisted per liter of 10 mM ammoniumformate with 0.1% formic acid in water and phase B same composition but then in methanol. At t=0 min 99% eluent A and 1% eluent B was used which changed gradually to 2% eluent A and 98% eluent B at 30 minutes. After each injection the sample was flushed using the start composition for 5 minutes. A calibration curve of vitamin B_{12} was prepared in order to be able to quantify B_{12} in each injection, after which final vitamin B_{12} contents were calculated using equation (6.1):

$$\begin{aligned} &Concentration_{product}(\frac{\mu g}{100g}) = \\ &Concentration_{detected}(\frac{\mu g}{ml}) * \frac{total\ extraction\ volume}{volume\ run\ over\ column} * \frac{100}{sample(g)} \end{aligned} \tag{6.1}$$

Dairy miso

Materials and methods for the dairy miso case study are available at: Dank, Mastrigt, Yang, et al. (2021)

Appendix A. Supplementary data

Supplementary material for Dank, Mastrigt, Yang, et al. (2021) is available online at: https://doi.org/10.1016/j.lwt.2021.111041

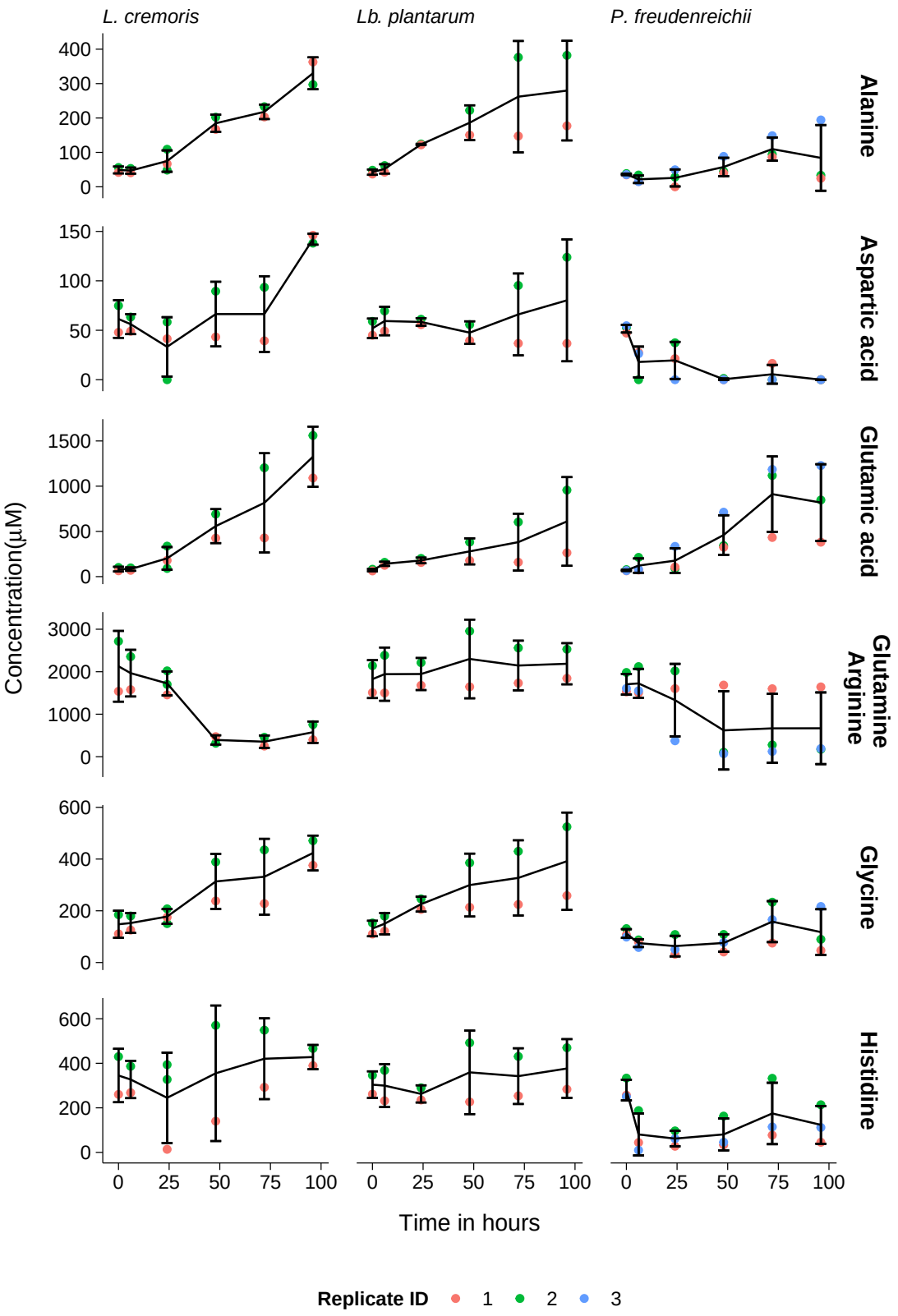

Figure 6.14: Free amino acid profiles during lupin quark production

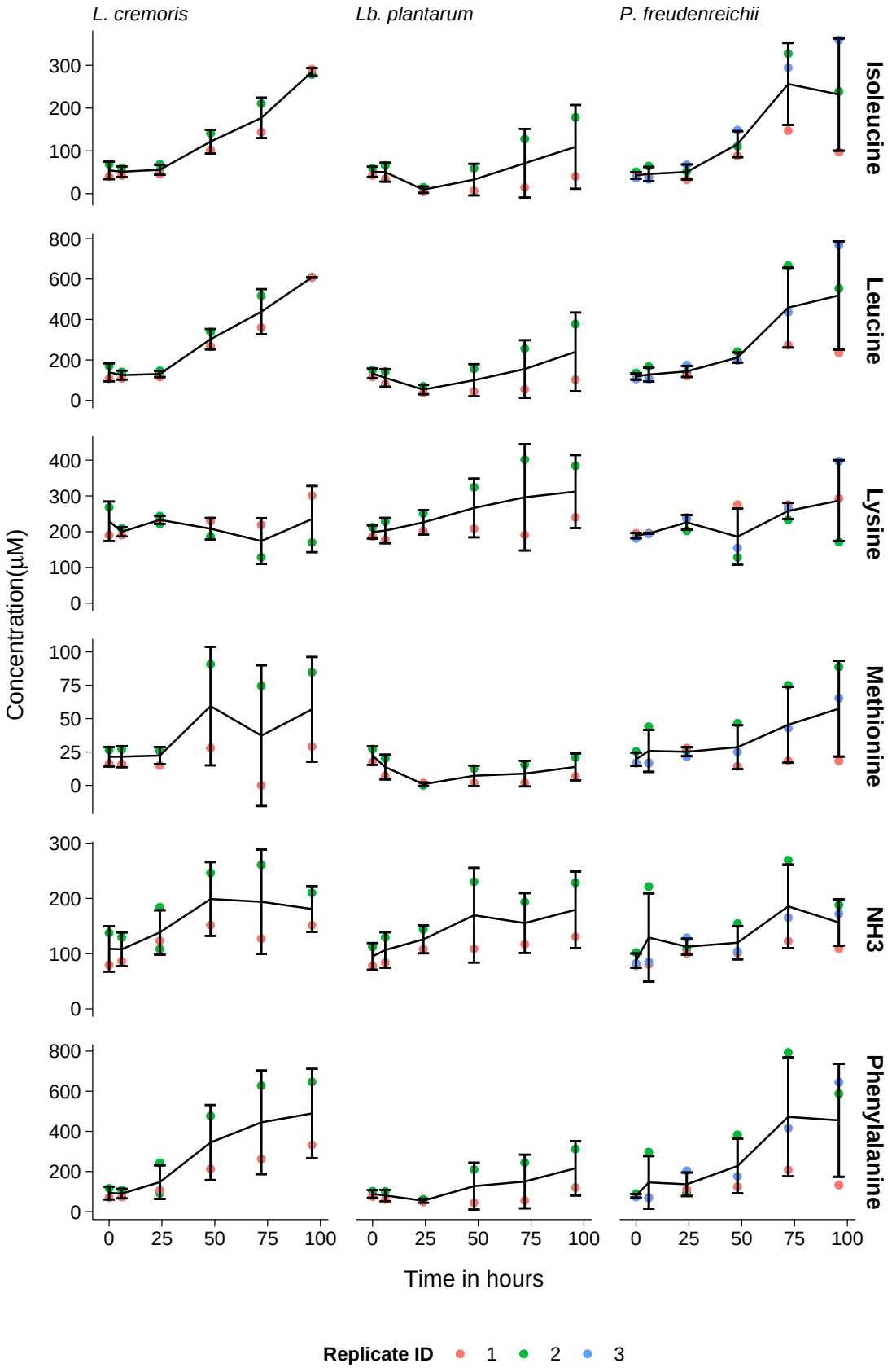

Figure 6.15: Free amino acid profiles during lupin quark production

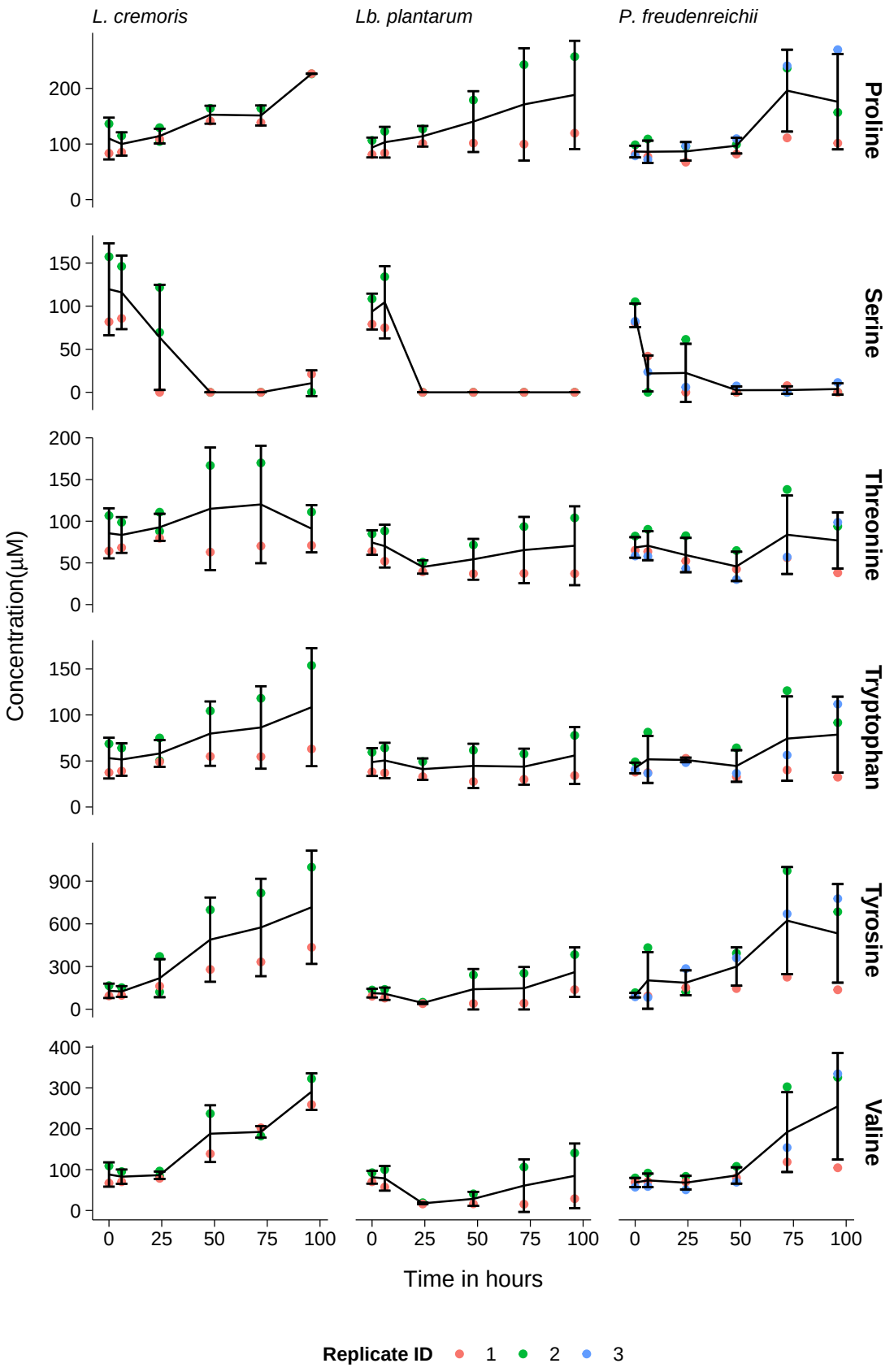

Figure 6.16: Free amino acid profiles during lupin quark production

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7
General discussion

A. Dank (2022)

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Z. Zeng, A. Dank, E. J. Smid, R. A. Notebaart, and T. Abee (2022). Bacterial microcompartments in food-related microbes. *Current Opinion in Food Science* 43:128–135

More than a century ago, in 1906, *Propionibacterium freudenreichii* was first described in Swiss Emmental cheese by Freudenreich and Orla-Jensen (1906). Not surprisingly, the majority of *P. freudenreichii* isolates available in international culture collections today are of dairy origin (Thierry et al. 2011). However, by itself *P. freudenreichii* grows poorly in milk at low cell numbers (Piveteau et al. 2000) and cannot be considered a milk adapted species (Falentin et al. 2010), which is also reflected by the inability to ferment lactose for some 'dairy' propionibacteria (Piveteau 1999). However, the presence of *P. freudenreichii* and prevalence in dairy products is not coincidental. Its low proteolytic activity (Thierry et al. 2011) and preference of lactate over glucose (Lee et al. 1974) points toward a lifestyle as consortium member of an environment in which proteolytic lactic acid bacteria or other lactate-producers are present. Of course, in cheese making lactose is converted by starter lactic acid bacteria to lactate, whilst casein molecules are degraded by rennet and lactic acid bacteria (Baer 1995), making a suitable environment for *P. freudenreichii* to survive and grow up to densities of 10^8 CFU per gram cheese (Beuvier et al. 1997).

The most likely source of *P. freudenreichii* in dairy products is as contaminant from the rumen or gut of the respective animal, as propionibacteria can be found in both sheep and cattle (Gutierrez 1953). Hence, it seems a niche for *P. freudenreichii* is in fact the colon environment found in a variety of animals, which also opens up potential for *P. freudenreichii* as human probiotic. A variety of evidence for survival in humans is known; *P. freudenreichii* is able to survive and remain metabolically active in the human gut (Hervé et al. 2007) and can adhere to host mucus (Ouwehand et al. 2000; Ouwehand et al. 2002; Tuomola et al. 1999) and host cells (Huang and Adams 2003). Furthermore, *P. freudenreichii* has been isolated from a breast-fed human infant (Colliou et al. 2017). This thesis focuses on *P. freudenreichii* as a potential gut microbe. A variety of evidence supporting a niche occupation in the gut or rumen of animals is provided (chapter 2, chapter 3, chapter 4). This knowledge consequently can be used in biotechnological applications, such as vitamin B_{12} production in bioreactor cultivations (chapter 5) or in novel fermented food

applications (chapter 6). A graphical overview of the main findings of this thesis and possible implications for biotechnological applications is displayed in Figure 7.1.

P. freudenreichii is adapted for growth in gastrointestinal tracts

Propionibacteria are present in the rumen of sheep (Mackie and Gilchrist 1979; Roxas 1980) and cows (Davidson 1998; Oshio et al. 1987), in the gut of pigs (Collado and Sanz 2007; Niu et al. 2015; Wang et al. 2018), chickens (Argañaraz-Martínez et al. 2013; Collado and Sanz 2007; Lee et al. 2019; Pourabedin et al. 2015; Wen et al. 2021), humans (Colliou et al. 2017; Dewulf et al. 2013) and even cockroaches (Cruden and Markovetz 1987). As mentioned previously, there is multiple evidence of survival and adherence to hosts by *P. freudenreichii* (Huang and Adams 2003; Ouwehand et al. 2000; Ouwehand et al. 2002; Tuomola et al. 1999). It therefor seems plausible that *P. freudenreichii* also occupies a niche within the gut microbiota of a variety of specific animals. To thrive within complex bacterial communities such as gut microbiota, one needs to be able to find suitable substrates and compete for these substrates with other consortium members. Based on the preference of lactate over more complex sugars, its low proteolytic activity and ability to utilize 1,2-propanediol and ethylene glycol (discussed below) it seems *P. freudenreichii* is specialized in degrading metabolic intermediates or end-products of other microbes present in its environment.

The role of bacterial microcompartments in *P. freudenreichii* and implications on interactions in the microbiome

A potential important substrate in the gut environment is 1,2-propanediol (1,2-PD). 1,2-PD is an anaerobic metabolic end-product of the metabolism of L-rhamnose or fucose by other intestinal microbiota such as *Escherichia coli* and *Bifidobacterium breve* (Cheng et al. 2020). Anaerobic metabolism of 1,2-PD generates the volatile toxic intermediate propionaldehyde. To reduce oxidative stress and to retain this volatile intermediate bacteria can produce proteinaceous organelles called bacterial

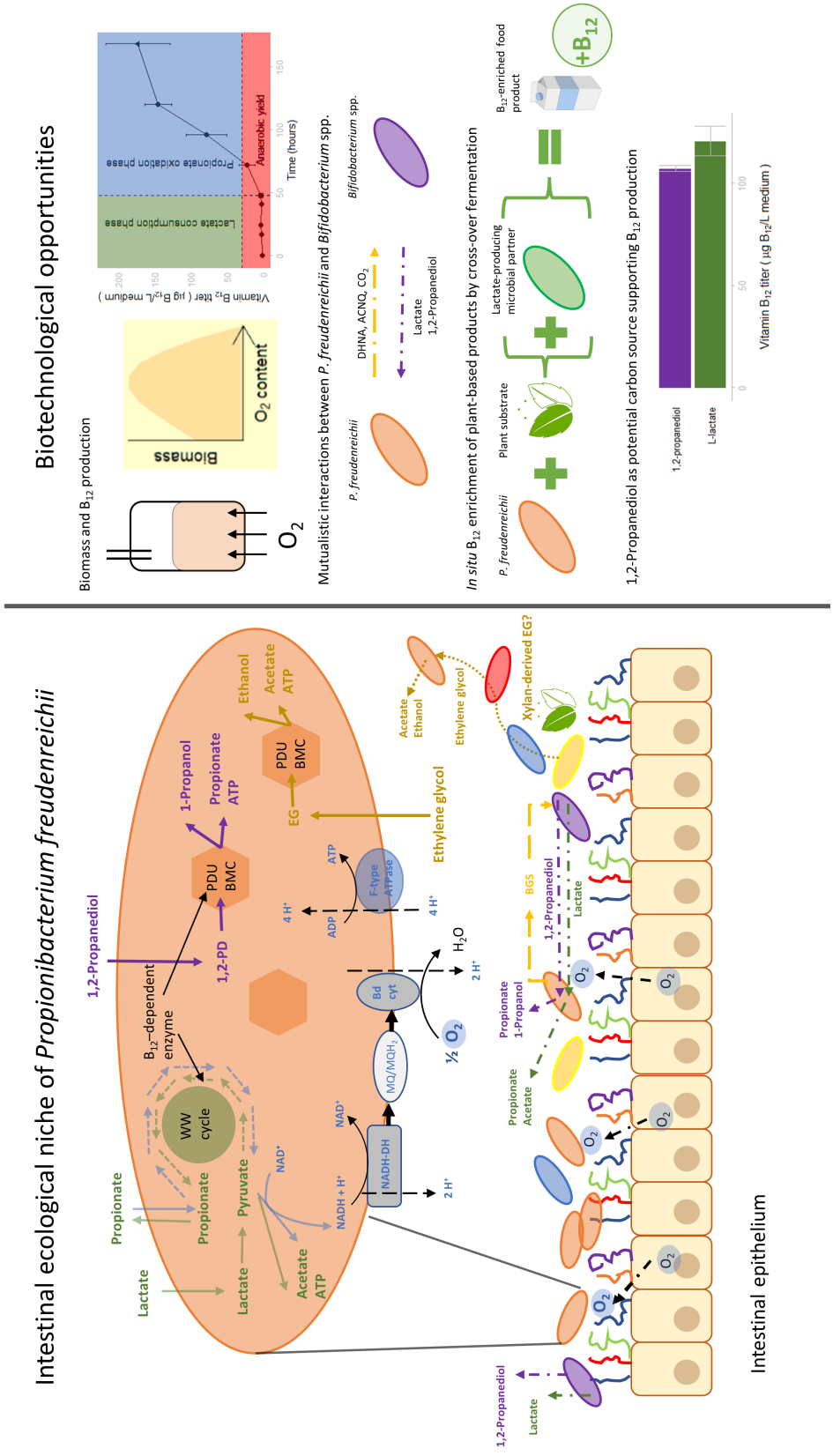

Figure 7.1: Graphical overview of main findings on *P. freudenreichii* metabolism and physiology of this thesis and implications on biotechnological applications

microcompartments (BMCs) (Cheng et al. 2008; Penrod and Roth 2006; Sutter et al. 2021). Hence, BMCs confer a fitness effect by enabling and facilitating more efficient use of 1,2-PD as a substrate.

L-Rhamnose is an abundant monosaccharide present in plant polymers within plant cell walls, such as pectin (Jiang et al. 2021). L-rhamnose can be liberated as monomer by activity of a variety of bacteria on rhamnogalacturonan (Avila et al. 2009; Laatu and Condemine 2003; Ochiai et al. 2007). L-rhamnose is metabolized to lactaldehyde which can be converted to lactate or 1,2-propanediol (Rodionova et al. 2013). L-fucose is an abundant monosaccharide present in glycolipids produced by mammalian cells and often exists as terminal sugar on glycan structures (Becker and Lowe 2003). Glycans containing fucose are abundantly expressed by intestinal epithelial cells (Becker and Lowe 2003; Torres-Pinedo and Mahmood 1984) and expression of these glycans aids in developing a healthy microbiome of nonharmfull commensal bacteria able to utilize fucose (Hooper et al. 2000). Indeed, syntrophic reactions between mucus-degrading bacteria and non-mucus degrading bacteria are reported (Belzer et al. 2017), showing the importance of trophic interactions in the gut microbiome. *Bifidobacterium bifidum* was shown to degrade mucus and deliver monosaccharides for utilization by other gut microbes such as *Eubacterium halii* (Bunesova et al. 2018; Schwab et al. 2017) and *Limosilactobacillus reuteri* (Cheng et al. 2020). *B. bifidum* releases lactate and acetate from the lactose moieties and releases 1,2-PD from the L-fucose moieties of mucus glycans (Cheng et al. 2020; Schwab et al. 2017). Both of these metabolic degradation products of mucus glycans provide suitable carbon sources for *P. freudenreichii*, showing great potential for mutualistic growth of *P. freudenreichii* and mucus-degrading bacteria (discussed below). Indeed, the ability to utilize mucus-derived 1,2-PD by expression of the *pdu* cluster was shown to be advantageous for growth in *Limosilactobacillus reuteri* (Cheng et al. 2020; Rattanaprasert et al. 2014), showing the potential fitness gain of the ability to utilize 1,2-PD in host guts.

We showed *P. freudenreichii* encodes a *pdu* cluster which is activated by the presence of 1,2-PD. The presence of 1,2-PD upregulates the protein expression of the

pdu cluster proteins, resulting in assembly of functional BMCs, metabolism of 1,2-PD to propionate and 1-propanol and additional biomass formation (chapter 3). Next to the release of propionate by metabolizing lactate through the Wood-Werkman cycle, metabolism of 1,2-PD by *P. freudenreichii* thus potentially contributes to delivery of health-promoting short chain fatty acids such as propionate (Zeng et al. 2022), which is also hypothesized to be the case for *pdu* encoding *L. reuteri* (Cheng et al. 2020). Furthermore, the *pdu* encoded PduCDE diol dehydratase requires vitamin B_{12} as cofactor and vitamin B_{12} is actively produced during 1,2-PD metabolism in *P. freudenreichii* (chapter 3), which could play an important role in cross-feeding (Belzer et al. 2017) and potentially aid in delivery of vitamin B_{12} to hosts (although this would require active transport of B_{12} or cell lysis).

The ability of *P. freudenreichii* to scavenge 1,2-PD and lactate allows potentially for a great mutualistic relationship with bifidobacteria. *Bifidobacterium* spp. release lactate and 1,2-PD from mucus-degrading activity, allowing a steady flux of suitable carbon sources to *P. freudenreichii*. *P. freudenreichii* did not show a preference for one of the two substrates in mixed-substrate fermentations (chapter 3), meaning both substrates could be metabolized simultaneously. This was also shown for *L. reuteri*, where glucose and 1,2-PD were co-metabolized and 1,2-PD was mainly used as electron acceptor (Cheng et al. 2020). These results were not found for the lactate-1,2-PD combination in *P. freudenreichii*, where substrate interactions were not apparent (chapter 3). Whether this is also the case for growth on sugars remains to be elucidated.

The positive association of the presence of *P. freudenreichii* and bifidobacteria has been well established. *P. freudenreichii* produces bifidogenic growth factors (BGS) such as 1,4-dihydroxy-2-naphtoic acid (DHNA) (Isawa et al. 2002; Kouya et al. 2007) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) (Kaneko 1999; Mori et al. 1997) and was also shown to enhance other gut microbes by BGS such as *Enterococcus faecalis, Bacteroides* spp. and *Enterobacter* spp. (Kaneko et al. 1994). *P. freudenreichii* is able to modulate and increase the amounts of *Bifidobacterium* spp. in humans (Bouglé et al. 1999; HoJo et al. 2002; Roland

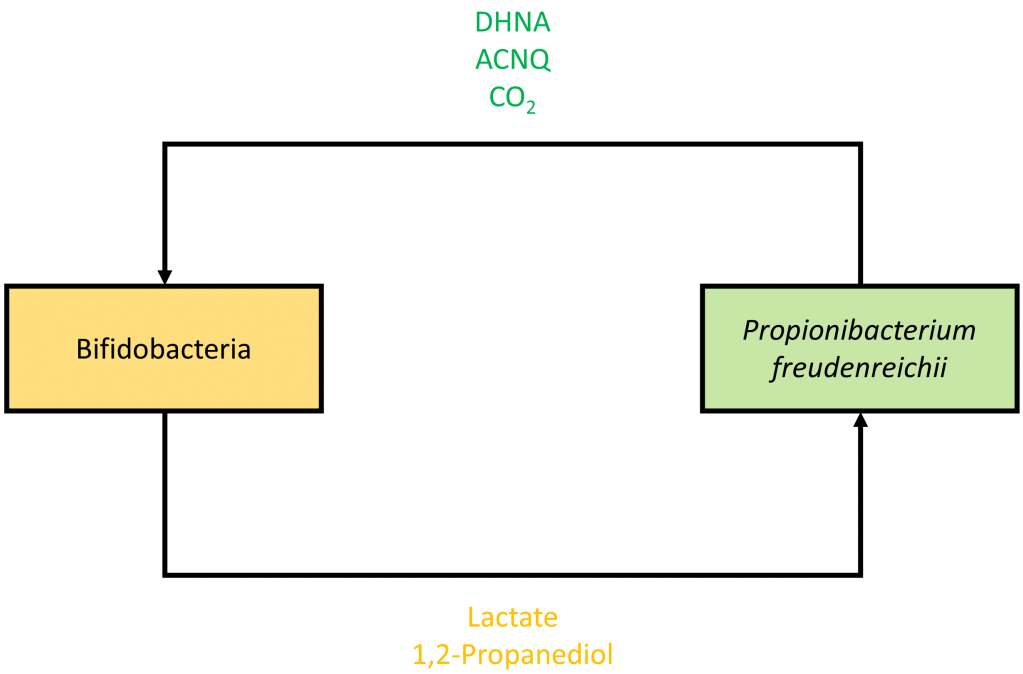

Figure 7.2: Mutualistic interactions between bifidobacteria and *P. freudenreichii*

et al. 1998). It is thus evident *P. freudenreichii* and bifidobacteria can greatly benefit from each other (see Figure 7.2).

The constant flow of carbon from host-derived glycans delivered by *Bifidobacterium* shows great potential for growth of *P. freudenreichii* and growth experiments with co-cultures of *Bifidobacterium* spp. and *P. freudenreichii* on mucus-derived media need to be performed to study this interesting interaction. As there is evidence of other bacteria being simulated by the BGS produced by *P. freudenreichii* it is suggested any of these microbes potentially can engage a mutualistic relationship with *P. freudenreichii* by delivering either lactate and/or 1,2-PD as carbon source. Next to potential positive interactions of course the ability to utilize 1,2-PD also allows direct competition against *pdu* encoding pathogens such as *Salmonella* and *Listeria monocytogenes*. The competition between commensal or beneficial *pdu* encoding organisms, such as *P. freudenreichii*, thus could play a therapeutic role against pathogens in the gut microbiome (Cheng et al. 2020; Zeng et al. 2022).

Another interesting metabolic intermediate potentially present in the gut is ethylene glycol (1,2-ethanediol, EG). EG is a potential metabolic end-product of

plant-cell wall degradation. EG may be produced from hemicellulose in anaerobic conditions by degradation of a consortium of microbes present in the microbiome (discussed in chapter 4). Interestingly, the drivers of hemicellulose degradation, i.e. *Bacteroides* spp. and *Prevotella* spp. (Chassard and Bernalier-Donadille 2006; Chassard et al. 2008; Chassard et al. 2007; Dodd et al. 2010; Salyers et al. 1982; Salyers et al. 1981), are key species in determining our microbiome fingerprint, our enterotype (Arumugam et al. 2011; Siezen and Kleerebezem 2011), and hence they are the main drivers in determining the importance of specic trophic chains. The enterotype dominated by *Bacteroides* spp. seems to be driven by fermentation of carbohydrates, whereas the enterotype dominated by *Prevotella* spp. is more driven by host-glycoprotein degradation and the enterotype driven by *Ruminococcus* spp. is driven by host-glycan degradation (Arumugam et al. 2011; Siezen and Kleerebezem 2011). The trophic chains are thus largely determined by the consortium member able to degrade the most-complex structures into more accessible nutrients. Thriving in one of these trophic chains thus requires enzymatic capabilities matching cross-feeding metabolites released by microbes higher-up in these chains. The ability to utilize EG as additional carbon source in anaerobic conditions may be such a beneficial trait. In chapter 4 we showed EG is metabolized by *P. freudenreichii* to ethanol and acetate, providing additional ATP. Addition of EG to the medium results in high upregulation of self-assembling PDU proteins, allowing formation of bacterial microcompartments and thus encapsulation of the metabolic pathway. Upregulation of the *pdu* cluster indicates a potential dual functionality of the *pdu* cluster in metabolizing both 1,2-PD and EG. The ability to metabolize not only 1,2-PD but also additional substrates such as EG by encapsulated pathways operating in BMCs allows for extension of the metabolic repertoire, increasing the potential for niche occupation in a variety of enterotypes. In case of a host-derived glycan entereotype the *pdu* cluster enables metabolism of 1,2-PD, at the end of the trophic chain of glycan degradation, whereas in an enterotype dominated by plant carbohydrate metabolism an addition functionality of EG metabolism would increase microbial fitness. A dual function of the *pdu* cluster would thus increase

the metabolic flexibility in terms of carbon utilization and thus potential to survive or even colonize in such environments, which is reported for 1,2-PD (Harvey et al. 2011) but remains to be validated for EG. In chapter 4 we discussed multiple bacteria with experimental evidence for the anaerobic degradation pathway of EG, but lacking evidence for the involvement of a functional BMC encoded by the *pdu* cluster. Based on whole genome sequences and the experimental evidence of EG metabolism it is very plausible the utilization of EG by these *pdu* cluster encoding organisms is mediated by BMCs. The *pdu* cluster therefor may have a broad-range distribution as dual functioning bacterial organelle.

(An)aerobic respiration and its implications for gut survival

One of the key characteristics of *P. freudenreichii* is the ability to metabolize lactate through the Wood-Werkman cycle (Thierry et al. 2011). Lactate is a common end product of bacterial fermentation in the gut by bifidobacteria and other lactic acid bacteria and is also formed by Bacteroidetes and Firmicutes (Flint et al. 2015). Feacal concentrations of lactate in healthy human subjects are usually low, as lactate is an important cross-feeding metabolite utilized by a variety of gut microbes (Flint et al. 2015). *P. freudenreichii* is also able to feed on lactate in anaerobic conditions by converting it to acetate and propionate. Lactate is metabolized to pyruvate after which it is either oxidized to acetate, generating 1 ATP by susbtrate level phosphorylation, or reduced to propionate using the Wood-werkman cycle. In the Wood-werkman cycle pyruvate is converted to fumarate, which is used as an electron acceptor through an anaerobic respiratory chain and respired to succinate, resulting in translocation of H^+ and consequent production of ATP through ATPase (Brooijmans 2008; McCubbin et al. 2020).

Fumarate acts as important electron acceptor in anaerobic conditions. Fumarate also was shown to be important for colonization of *E. coli* in the murine intestines (Jones et al. 2007; Jones et al. 2011). Fumarate itself is normally not present in high amounts in the environment. However, L-aspartate can be found in higher amounts in the murine intestine (Schubert et al. 2021). L-aspartate can be converted

to fumarate and NH_3 by aspartate ammonia lyase. By this way, L-aspartate acts as important source of fumarate and thus acts as main source of anaerobic fumarate respiration (Schubert et al. 2021). In *E. coli* the ability to utilize aspartate for fumarate respiration was shown to contribute significantly to survival and colonization of murine (Schubert et al. 2021) and bovine (Bertin et al. 2018) intestines. Next to providing fumarate for respiration, the release of ammonia also significantly contributes to the nitrogen requirement of *E. coli* (Schubert et al. 2021). *P. freudenreichii* also encodes aspartate ammonia lyase and was found to metabolize aspartate to fumarate and utilize it as electron acceptor (Blasco et al. 2011; Crow 1986; Crow 1987). The released NH_3 can act as nitrogen source for *P. freudenreichii* as well, as it is able to synthesise all amino acids *de novo* (Falentin et al. 2010). It is therefore very plausible that just as in *E. coli*, for *P. freudenreichii* L-asparate availability in the colonic environment plays an important role as source of electron acceptor and source of nitrogen.

Next to L-aspartate, the ability to utilize other electron acceptors will provide fitness benefits. The most potent electron acceptor is of course oxygen. The gastro-intestinal tract of animals (Hillman et al. 1989) and humans (Espey 2013) are not devoid of oxygen. Oxygen is delivered from colonic tissue to the intestinal lumen (Albenberg et al. 2014). A consortium of facultative anaerobic and aerotolerant bacteria consequently consume this oxygen (Albenberg et al. 2014; Morris and Schmidt 2013), resulting in a zone with O_2 concentrations decreasing from oxic to anoxic. Since the presence of oxygen allows aerobic respiration, which is energetically more favourable than fermentation, competition for this electron acceptor can be anticipated in niches where it is not highly abundant (Fenchel and Finlay 2008). Not surprisingly, high-affinity oxidase genes are important for the microbiota in the mammalian gastro-intestinal tract (Morris and Schmidt 2013) and prove important for colonization of *E. coli* and *Campylobacter jejuni* in mouse (Jones et al. 2007) and chicken intestines (Weingarten et al. 2008) respectively (Morris and Schmidt 2013). One such high-affinity oxidase is cytochrome-bd (Baughn and Malamy 2004; D'mello et al. 1996). *P. freudenreichii* encodes cytochrome bd-oxidase (Falentin

et al. 2010; Koskinen et al. 2015) and expresses it in anaerobic and microaerobic conditions (chapter 2). By utilizing low amounts of oxygen, *P. freudenreichii* can benefit greatly in terms of energy production and is able to oxidize its fermentation products propionate and acetate (chapter 2, chapter 5). Interestingly, fumarate metabolism also allows propionate consumption in *P. freudenreichii* (Rosner and Schink 1990). *P. freudenreichii* can thus choose from a range of metabolic options depending on substrates and the presence of aerobic or anaerobic electron acceptors, showing its great metabolic flexibility. The ability to have respiratory flexibility in the intestinal tract greatly increases fitness of bacteria by maximizing energetic efficiency by respiration (Jones et al. 2007).

Based on the utilization of metabolic end products with relative low energetic potential such as lactate, 1,2-PD and EG and the flexibility between utilization of asparate for anaerobic fumarate respiration and aerobic respiratory capabilities using a high-affinity cytochrome bd-oxidase suitable for respiration at low oxygen tensions it seems *P. freudenreichii* is well adapted for growth in intestinal environments.

Biotechnological applications

In chapter 2 we have shown that oxygen is a potent electron acceptor at low oxygen levels and controlling the amount of oxygen allows for high increments of biomass production, which may be of use for production of starter/adjunct cultures. *P. freudenreichii* is able to completely oxidize lactate in three distinct phases, thereby increasing energetic efficiency from lactate and acchieving higher biomass yields (~2.5-fold higher, chapter 2, chapter 5). Higher biomass formation not only increases biomass yield per substrate, but may also be a sensible strategy for the production of vitamin B_{12}, as this may be (partly) coupled to biomass production. Furthermore, oxidation of organic acids reduces potential product inhibition effects (Pinhal et al. 2019; Wang et al. 2010; Ye et al. 1996) and may prevent decreasing growth rates due to organic acid, and especially propionate, accumulation (Nanba et al. 1983; Ye et al. 1996). Microaerobic growth conditions thus show great potential for production of biomass and vitamin B_{12} by *P. freudenreichii*. In chapter 5 the

potency of oxygen in vitamin B_{12} production was shown, as oxidation of lactate increased B_{12} yield per lactate 4.5 times. Furthermore, it was revealed this may be largely linked to the phase in which propionate is oxidized. A fed-batch reactor experiment confirmed propionate supported biomass formation and B_{12} production and a 26.3 times higher yield per mmol of propionate compared to yield per mmol of lactate in anaerobic conditions was obtained. Furthermore B_{12} production per cell also drastically increased compared to anaerobic conditions. Oxygen availability thus may also play a crucial role for vitamin B_{12} production by *P. freudenreichii* in food fermentations. In solid-state fermentations, such as cheese ripening, diffusion of oxygen into the food matrix may be a key factor in determining the final *P. freudenreichii* population density (Reinbold et al. 1958) and hence B_{12} production. In sunflower-seed milk, *P. freudenreichii* was shown to oxidize propionate and to produce more B_{12} at specific oxygen regimes (Tangyu et al. 2022). However, the range at which oxygen is beneficial seems to be small and therefore this factor needs to be strictly controlled, for instance by controlling oxygen flux in production tanks or spatial dimensions such as product thickness (Dank et al. 2021; Pritchard et al. 1977; Tangyu et al. 2022).

Its preference for lactate over complex sugars, the ability to degrade 1,2-propanediol and the low nutritional requirements (Falentin et al. 2010; McCubbin et al. 2020) reflects the niche of *P. freudenreichii* at the lower end of trophic chains in microbial communities. Due to its low nutritional requirements, a great potential lies within applications of *P. freudenreichii* in cross-over food fermentations and especially in combination with other (proteolytic) lactate-producing organisms. Cross-over fermentations are fermentations in which a microbe is introduced to a novel substrate and/or microbial partner (chapter 6), thereby creating a product with enhanced nutritional or organoleptic properties. Of much interest is nutritionally fortifying plant-based products with B_{12} by including *P. freudenreichii* in the fermentation consortium. Examples of successful *in situ* B_{12} fortification by cross-over fermentation include tempeh (Wolkers–Rooijackers et al. 2018), a variety of cereals, brans and legume flour (Wang et al. 2022; Xie et al. 2021; Xie et al.

2019), sunflower seed milk (Tangyu et al. 2022) and kefir grains (Van Wyk et al. 2011). Most often *P. freudenreichii* is combined with lactic acid bacteria, but also successful combinations with *Bacillus amyloliquefaciens* (Tangyu et al. 2022) and the lactate-producing fungus *Rhizopus oryzae* (Wolkers–Rooijackers et al. 2018) are possible. Next to lactate, 1,2-PD also supports B_{12} production in *P. freudenreichii* (chapter 3). 1,2-PD is an approved food-additive (Food Additives et al. 2018) and is applied in a range of products including beverages and bread. Furthermore, 1,2-PD may be produced *in situ* by including a 1,2-PD-producing consortium partner such as *Lentilactobacillus buchneri*, as exemplified in sourdough bread (Zhang et al. 2010). However, studies focusing on the effect of 1,2-PD supplementation and B_{12} enrichment by *P. freudenreichii* in food fermentations should be performed to validate this hypothesis.

Conclusions

Recent evidence links *P. freudenreichii* to a niche in the GI-tract of animals. This is reflected by its capacity to grow in anaerobic and microaerobic conditions using fermentative and respiratory metabolism with oxygen and fumarate acting as terminal electron acceptor, and the ability to utilize typical gut substrates such as 1,2-PD. Due to its low nutritional requirements and the ability to produce the active form of B_{12}, *P. freudenreichii* can be applied in the production of novel fermented food products, especially in plant-based fermentations. Optimizations of these novel fermentations can be done by choosing the correct consortium partners and taking novel insights in ecophysiological behavior of *P. freudenreichii* in mind. Its evidence as probiotic species further promotes the use of *P. freudenreichii* in novel healthy and probiotic food fermentations.

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8
Summary

Summary

Propionibacterium freudenreichii is a propionic acid bacterium that has been isolated from Swiss-type cheese. *P. freudenreichii* is well-known for its ability to ferment lactate into propionate, acetate and CO_2, thereby contributing to the formation of the characteristic holes (or 'eyes') found in Swiss-type cheeses. *P. freudenreichii* is mainly studied for three different reasons; I) The production of cheese, II) the production of propionic acid, III) the production of vitamin B_{12}. In recent years there is also an increasing interest in the prebiotic (bifidogenic) and probiotic potential of *P. freudenreichii*.

This thesis describes studies of *P. freudenreichii* focusing on its metabolic and physiological response to conditions it may encounter in mammalian guts. Two main aspects were studied; I) *P. freudenreichii* is considered anaerobic to aerotolerant, but encodes a complete TCA cycle and aerobic respiratory chain with a high-affinity terminal oxidase, II) *P. freudenreichii* encodes a **P**ropane**d**iol **u**tilization (*pdu*) cluster and thus has the potential to produce bacterial microcompartments. Both traits support a niche occupation of the gut, as both oxygen (in low amounts) and 1,2-propanediol (metabolized by the *pdu* cluster) are present in the gut. Lastly, the obtained knowledge on primary metabolism was used for biotechnological applications, such as the production of vitamin B_{12} *in situ* in a novel food product and in a bioreactor.

Oxygen serves as potent terminal electron acceptor at low oxygen fluxes but is toxic at higher fluxes for *P. freudenreichii*

In chapter 2 a variety of propionic acid bacteria were screened for their response to 3 cultivation regimes, namely anaerobic conditions, aerobic static conditions and aerobic shaking conditions. In general, the lowest mean biomass formation was found in anaerobic conditions. Aerobic static conditions were found to have the highest mean biomass formation. Aerobic shaking conditions also increased mean biomass formation but also enlarged the distribution of biomass levels found. These results clearly show that oxygen has the potential to be energetically favourable

for propionic acid bacteria and hence can be used as terminal electron acceptor, but is also toxic at higher levels. Toxicity and potential to form more biomass was strain-dependent. An in-depth study was performed on *P. freudenreichii* DSM 20271 in a chemostat, in which at a set growth rate the oxygen flow was gradually increased. Increasing the oxygen flow resulted first in higher biomass formation. Oxygen was increased until it started to become deleterious with biomass levels dropping again, indicating indeed an optimal oxygen flow can be found for this strain. Interestingly, fermentation end-products remained to be found. To study its metabolism in more depth, batch cultivations using the 'optimal' oxygen flow were performed. It was found *P. freudenreichii* was able to completely respire lactate in three distinct phases. First lactate was consumed and largely fermented to propionate and acetate. When lactate was depleted, propionate was respired to acetate and lastly when propionate was depleted acetate was respired. The observed phases likely result from a stochiometric limitation of oxygen and hence selects for optimal uptake of potential substrate (lactate) and optimal energetic benefit once this is depleted (respiration of propionate to acetate requires less oxygen then complete respiration to CO_2). A proteome analysis revealed *P. freudenreichii* expresses aerobic electron transport chains in anaerobic and microaerobic conditions, indicating it has the metabolic flexibility to change from fermentation to respiration once oxygen comes available. This links to an environmental niche where oxygen is scarce and competition between this electron acceptor arises, such as microoxic zones like those found in the gut and in soils.

P. freudenreichii encodes a functional *pdu* cluster supporting a niche occupation of gut environments

Based on earlier results found in chapter 2 leading to the hypothesis of *P. freudenreichii* occupying a niche in gut environments and recent evidence of *P. freudenreichii* being able to survive and colonize gut environments I aimed to identify potential other traits supporting evidence of this niche occupation. In chapter 3 we identified the *pdu* cluster to be encoded in the genome of *P. freudenreichii*.

Summary

The *pdu* cluster encodes a bacterial microcompartment (BMC) implicated with the metabolism of 1,2-propanediol. 1,2-Propanediol is a metabolic end-product from microbial fermentation of sugars from dietary fiber and host glycans, such as rhamnose and fucose. BMCs enable metabolism of substrates with metabolic pathways containing volatile or toxic intermediates, such as aldehydes, by encapsulating the catalytic enzymes by a protein shell. BMCs facilitate use of substrates by reducing toxicity and loss of volatile intermediates. In chapter 3 we found that 1,2-propanediol induces expression of BMCs, enabling metabolism of 1,2-propanediol to 1-propanol and propionate and supporting biomass and vitamin B_{12} formation. BMCs were visualized using transmission electron microscopy, providing evidence *P. freudenreichii* is able to form BMCs and metabolize 1,2-propanediol through a BMC-mediated pathway. Proteome analysis revealed significant upregulation of DNA and RNA repair pathways and taken together with observed loss of expected C_3 compounds it is conceivable propionaldehyde retention in the BMC is not optimal. Proteins required in lactate metabolism were downregulated, such as fumarate reductase and ATPase required for oxidative phosphorylation, signifying a metabolic switch on 1,2-propanediol.

The *pdu* cluster encodes for metabolism of multiple substrates

In other bacteria the *pdu* cluster has also been implicated with the metabolism of glycerol. Furthermore, in *Acetobacterium woodii* ethylene glycol (1,2-ethanediol) was found to be metabolized in bacterial microcompartments encoded by the *pdu* cluster. In chapter 4 we showed that also *P. freudenreichii* is able to metabolize ethylene glycol in anaerobic conditions. Metabolism of ethylene glycol leads to strong induction of the *pdu* cluster, resulting in metabolism of ethylene glycol to ethanol and acetate and corresponding increase of biomass formation. Furthermore, proteome analysis revealed upregulation of a variety of stress related proteins involved in DNA and RNA repair processes and upregulation of sulfur-metabolic pathways leading to cysteine and methionine. Comparative analysis of upregulated proteins

on 1,2-propanediol and ethylene glycol revealed this up regulation is associated with BMC-metabolism and thus BMC-metabolism requires higher expression of cellular repair processes of DNA, RNA and proteins. Based on metabolic evidence coupled to whole genome sequences of other microbes we discussed the possibility of the *pdu* cluster having a dual function in metabolism of 1,2-propanediol as well as ethylene glycol. Ethylene glycol is a potential metabolic end-product of xylan degradation and hence potential implications of the *pdu* cluster in relation to gut xylan metabolism were discussed.

Vitamin B_{12} production increases at low oxygen fluxes

The 'propionate switch' and corresponding higher biomass formation found in chapter 2 showed potential for vitamin B_{12} production. In chapter 5 we monitored biomass formation, metabolite production and *de novo* vitamin B_{12} production by *Propionibacterium freudenreichii* on a chemically defined medium using the microaerobic conditions used in chapter 2. Similarly as in chapter 2, biomass formation was found to increase more than 2-fold (2.7 times) on the same amount of lactate in microaerobic conditions compared to anaerobic conditions. The vitamin B_{12} titer was found to increase six-fold in microaerobic conditions compared to anaerobic conditions, showing the potential of small amounts of oxygen to be beneficial for vitamin B_{12} production. Consequently, a fed-batch reactor using small injections of propionate was run to produce vitamin B_{12} using propionate as carbon source. Vitamin yields increased from 0.3 μg B_{12} per mmol lactate in anaerobic conditions to 2.4 μg B_{12} per mmol lactate and 8.4 μg B_{12} per mmol propionate in microaerobic conditions. Yield per cell dry weight (CDW) increased from 41 μg per g CDW in anaerobic conditions on lactate to 92 μg per g CDW on lactate and 184 μg per g CDW on propionate in microaerobic conditions. Hence, propionate oxidation in microaerobic conditions shows great potential for efficient production of vitamin B_{12}.

Cross-over fermentation is an excellent tool for food product development

Cross-over fermentations are processes in which a microorganism is taken from a traditional fermentation process and is introduced onto a new substrate and/or introduced to a new microbial partner in a mixed culture. These fermentations can result in interesting novel food products with enhanced nutritional or organoleptic properties. in chapter 6 we exemplified cross-over fermentations by showing two examples; One example focuses on designing a novel food with intense aromatic properties by applying *Aspergillus oryzae* on quark and the other focuses on developing a B_{12} fortified lupin milk by using *P. freudenreichii*. Both examples clearly show interesting product characteristics can be accomplished by selecting potent microbes and applying them on unconventional substrates. Cross-over fermentation thus has great potential for novel food product design.

Conclusions

P. freudenreichii likely occupies a niche in the GI-tract of animals, as it has been isolated from a variety of animals and even from a human infant. Its niche occupation in the gut is reflected by its capacity to grow in anaerobic and microaerobic conditions using fermentative and respiratory metabolism with oxygen acting as terminal electron acceptor, and the ability to utilize typical gut substrates such as 1,2-PD and lactate. Due to its low nutritional requirements and the ability to produce the active form of B_{12}, *P. freudenreichii* can be applied in the production of novel fermented food products, especially in plant-based fermentations. Optimizations of these novel fermentations can be done by choosing the correct consortium partners and implementation of novel insights in ecophysiological behavior of *P. freudenreichii*. Evidence supporting the probiotic status of *P. freudenreichii* further promotes the use of *P. freudenreichii* in novel healthy and probiotic food fermentations.

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Alexander Dank

Katwijk aan Zee

16 april 2023

About the author

Alexander Dank was born on 14 March 1994 in Sprang-Capelle, the Netherlands. He started a bachelor life science and technology in 2012 at Wageningen University and switched to food technology in 2013. He completed his bachelor with a thesis entitled "*Enzymatic degradation of oligosaccharides from high and low glycosylated caseinate by probiotic bacteria*" in 2016. He started his MSc degree in food technology, specialisation food biotechnology and biorefining in 2016 at Wageningen University by participating in an Erasmus exchange program to KU Leuven. Alexander graduated *cum laude* in 2018 with a thesis entitled "*Modulating yeast aroma profiles using CRISPR-Cas genome editing"* and an intership at Arla Foods Amba, Denmark. In 2018 he continued at the Laboratory of Food Microbiology at Wageningen University and research with his PhD thesis entitled"*Propionibacterium freudenreichii*, more than meets the 'eyes'".

List of publications

- A. Dank, O. van Mastrigt, Z. Yang, V. M. Dinesh, S. K. Lillevang, C. Weij, and E. J. Smid (2021). The cross-over fermentation concept and its application in a novel food product: the dairy miso case study. *LWT* 142:111041

- A. Dank, O. van Mastrigt, S. Boeren, S. K. Lillevang, T. Abee, and E. J. Smid (2021). *Propionibacterium freudenreichii* thrives in microaerobic conditions by complete oxidation of lactate to CO_2. *Environmental Microbiology*

- A. Dank, Z. Zeng, S. Boeren, R. A. Notebaart, E. J. Smid, and T. Abee (2021). Bacterial microcompartment-dependent 1,2-propanediol utilization of *Propionibacterium freudenreichii*. *Frontiers in microbiology* 12

- Z. Zeng, A. Dank, E. J. Smid, R. A. Notebaart, and T. Abee (2022). Bacterial microcompartments in food-related microbes. *Current Opinion in Food Science* 43:128–135

- A. Dank, G. Biel, T. Abee, and E. J. Smid (2022). Microaerobic metabolism of lactate and propionate enhances vitamin b12 production in *Propionibacterium freudenreichii*. *Microbial cell factories* 21(1):1–10

- A. Dank, E. J. Smid, and R. A. Notebaart (2018). Crispr-cas genome engineering of esterase activity in saccharomyces cerevisiae steers aroma formation. *BMC research notes* 11(1):1–6

Overview of training activities

Discipline specific activities

Courses

Course	Organizer	Year
Advanced Proteomics	Wageningen University & Research, laboratory of Biochemistry	2019
Innovation Towards Plant-Based Consumption	Department of Food Science, University of Copenhagen	2021
Giving lecture at Ecophysiology of food-related microbes	VLAG	2021

Meetings

Scientific meeting	Organizer	Year
De complexe wereld van gefermenteerde zuivel Najaarssymposium 2017	Genootschap ter bevordering van melkkunde	2017
The Brave New World of Smart Data & Semantics in the Life Sciences	Laboratory of Systems and synthethic biology, WUR	2018
FEMS scientific congress 2019 + poster presentation	FEMS	2019
ARLA-WUR project meetings	ARLA, WUR	2018-2022
Presentations PhD week China	WUR FHM	2019
WEES Symposium lecture	WEES	2019
Young EFFoST day 2019	Young EFFoST	2019
33rd EFFoST international conference	EFFoST	2019
1st Wageningen Food Science Symposium + oral presentation	WUR/VLAG	2019
Webinar: Ensuring Microbiological Quality and Safety of B2B and Finished Products	NIZO	2020
National Biotechnology Congress NL	Nationale biotechnology vereniging (NBV)	2022

General Courses

Course	Organizer	Year
VLAG PhD week	VLAG	2018
Scientific Publishing	WGS	2018
Scientific Artwork, Data visualisation and infographics with Adobe illustrator	WUR Library	2020
Reviewing a scientific manuscript	WGS	2021
Carreer Assessment	WGS	2021
Introduction to R	Datacamp	2019
Intermediate R	Datacamp	2019
Cleaning data in R	Datacamp	2019
Data Manipulation in R with dplyr	Datacamp	2019
Joining data in R using dplyr	Datacamp	2019
Visualisation best practices in R	Datacamp	2019
Data Visualization with ggplot 2 (Part 1, Part 2 & Part 3)	Datacamp	2019
Introduction to data	Datacamp	2019
Exploratory data analysis	Datacamp	2019
Correlation and Regression	Datacamp	2019
Multiple and logistic regression	Datacamp	2019
Experimental design in R	Datacamp	2019
Introduction to writing functions in R	Datacamp	2020
Applied statistics in R	Biometris, VLAG	2019
Introduction to python	Datacamp	2020
Intermediate python	Datacamp	2020
Building Web Applications with Shiny in R.	Datacamp	2021
Writing efficient R code	Datacamp	2021

Additional activities

Activity	Location	Year
Preparation of research proposal	research group	
Weekly FHM group meetings	research group	
PhD Excursion china	FHM	2019
YoungWUR Futsal tournament (1st place)	YoungWUR	2019
Young EFFoST day 2019 organising committee		2019
Lab outing FHM 2020		2020

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